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EXPERIMENTAL MEDICINE

EDITED BY
SIMON FLEXNER, M.D.

VOLUME THIRTY-FIRST
WITH NINETY-SEVEN PLATES AND EIGHTY-ONE
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THE LYMPHOCYTE IN NATURAL AND INDUCED RESISTANCE TO TRANSPLANTED CANCER.

V. HISTOLOGICAL STUDY OF THE LYMPHOID TISSUE OF MICE WITH INDUCED IMMUNITY TO TRANSPLANTED CANCER.

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PLATES 1 AND 2.

(Received for publication, October 29, 1919.)

A number of theories have been brought forward to explain the natural and induced resistance to the transplanted cancers of mice, but none of them has covered all the facts or met with general acceptance.

The so called athrepsia theory of Ehrlich¹ has been the most prominent of these explanations, but in the light of more recent work it apparently does not account for the phenomenon. This theory was based on the observation that mouse tumors inoculated into rats or *vice versa* were capable of survival and growth for a time but would die if allowed to remain. If, however, the graft was removed from the rat during the proliferative stage and then returned to the mouse it would continue to grow and after a period could again be transplanted to rats. This zigzag grafting could be continued indefinitely. The interpretation offered by Ehrlich was that each species produces a specific and limited amount of food substance necessary for growth (substance X). The temporary survival and growth of the mouse tumor in the rat was accounted for by the amount of substance X carried over with the graft, and when this was exhausted the mouse tissue died from lack of food. When the tumor was returned to the mouse it would accumulate a fresh supply of the substance and would be able again to live for a time in the rat. Lambert and Hanes² showed that a tissue of one species could live and grow for some time in the plasma of another. In fact, growth was almost as good as when the tissues were placed in homologous plasma. Murphy³ demonstrated that mammalian tissue could be carried through

¹ Ehrlich, P., *Arch. k. Inst. exp. Therap. Frankfurt*, 1906, No. 1, 84.

² Lambert, R. A., and Hanes, F. M., *J. Exp. Med.*, 1911, xiv, 129.

³ Murphy, Jas. B., *J. Exp. Med.*, 1913, xvii, 482.

many generations in the chick embryo without return to its native species. Furthermore, he showed that rats, when subjected to suitable exposures of x-rays, were capable of supporting a mouse tumor in active growth over prolonged periods with repeated transplantation.⁴ From the facts of these investigations the athrepsia theory does not seem to meet the conditions.

A theory, brought forward by Russell⁵ and strongly supported by the workers of the Imperial Cancer Research Fund, attributes cancer resistance to a failure of the stroma reaction. Thus, a graft in a resistant animal fails for lack of suitable blood supply and framework to support growth. This explanation has not met with wide acceptance, for it does not explain naturally acquired resistance. Moreover, a number of observers have described failure of cancer grafts in resistant animals in which there is an abundant vascular and stroma reaction (Burgess,⁶ Rous,⁷ Goldmann,⁸ Levin⁹).

Tyzzar,¹⁰ finding an identical histological reaction in resistant animals, whether the immunity was induced or natural, concludes that the same factors are responsible for both conditions. The chief difference is the time at which the reaction takes place, which is earlier in the animals with induced resistance than in those with natural resistance. He expresses the idea that the associated "inflammation" is the defensive factor. The reaction is characterized in the early stages by an accumulation of polymorphonuclear leucocytes around the graft, followed later by lymphocytic infiltration and an increase in the connective tissue elements. The first stage, the polymorphonuclear accumulation, is the same in the susceptible and resistant animals and so may be disregarded in a consideration of the immunity factors. Tyzzar suggests that the failure of the graft in these cases is due to a choking of the blood supply and a starvation of the introduced cells. The view we have favored is that the immunity is due to cellular reaction but that the lymphoid elements are the important agents in this process.

Da Fano¹¹ was the first to call attention to the lymphocyte as the possible active agent in cancer immunity. He noted the fact that there was not only an accumulation about the graft in resistant animals but also an increase in the numbers of these cells in the subcutaneous tissues. Baeslack¹² observed a percentage increase in the circulating lymphocytes in a very small series of immune

⁴ Murphy, Jas. B., *J. Am. Med. Assn.*, 1914, lxii, 1459.

⁵ Russell, B. R. G., in Bashford, E. F., *3rd Sc. Rep., Imperial Cancer Research Fund*, 1908, 341.

⁶ Burgess, A. M., *J. Med. Research*, 1909, xxi, 575.

⁷ Rous, F. P., *J. Am. Med. Assn.*, 1910, lv, 1805.

⁸ Goldmann, E. E., *Beitr. klin. Chir.*, 1911, lxxii, 1.

⁹ Levin, I., *J. Exp. Med.*, 1911, xliii, 604; xiv, 139.

¹⁰ Tyzzar, E. E., *J. Cancer Research*, 1916, i, 125.

¹¹ Da Fano, C., *Z. Immunitätsforsch., Orig.*, 1910, v, 1.

¹² Baeslack, F. W., *Z. Immunitätsforsch., Orig.*, 1913-14, xx, 421.

animals. Murphy and Morton¹³ first showed that there was an actual increase in the circulating lymphocytes in animals during the development of active resistance, both when induced and when naturally acquired. The workers in this laboratory have extended this work to show a close relation between the lymphocyte reaction and the resistance to cancer. Mice rendered potentially immune by the injection of blood can be made susceptible by the destruction of the lymphoid tissue.¹³ Likewise, mice of tested immunity, after suitable exposure to x-rays, become susceptible to inoculation.¹⁴ These observations of Murphy and his coworkers have been confirmed and extended by Mottram and Russ.¹⁵ Moreover, the former found that animals with an artificially induced lymphocytosis¹⁶ become as highly resistant to cancer implants as they do following tissue injection.¹⁷ In the latter experiment a parallelism was noted between the number of lymphocytes in the blood and the number of mitotic figures in the spleen and lymph glands.¹⁸

In the work presented in this paper a study has been made of the lymphoid organs in animals with induced immunity to cancer to establish a further link in the evidence associating the lymphocyte with cancer immunity and to ascertain if possible the source and nature of the blood lymphocytosis. In the course of the experiments a histological examination was made of the changes in the subcutaneous tissue in order to check and possibly extend the earlier observation of Da Fano.¹¹

Material.

The material for the study has been collected in the course of five experiments embracing over 100 mice, as described in the following protocols.

Experiment 1.—Thirty-four white mice were given an injection of 0.3 cc. of defibrinated mouse blood in the subcutaneous tissue of the back. Three mice were killed on the 6th day and three on the 10th day after the immunizing in-

¹³ Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 204.

¹⁴ Murphy, Jas. B., and Taylor, H. D., *J. Exp. Med.*, 1918, xxviii, 1.

¹⁵ Mottram, J. C., and Russ, S., *Proc. Roy. Soc. London, Series B*, 1917, xc, 1.

¹⁶ Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 800. Murphy, Jas. B., and Sturm, E., *J. Exp. Med.*, 1919, xxix, 1. Thomas, M. M., Taylor, H. D., and Witherbee, W. D., *J. Exp. Med.*, 1919, xxix, 75.

¹⁷ Murphy, Jas. B., and Sturm, E., *J. Exp. Med.*, 1919, xxix, 25. Russ, S., Chambers, H., Scott, G. M., and Mottram, J. C., *Lancet*, 1919, i, 692.

¹⁸ Nakahara, W., *J. Exp. Med.*, 1919, xxix, 17, 83.

jection. The remaining twenty-eight mice were inoculated, 10 days after the blood injection, with bits of Bashford Adenocarcinoma No. 63. They were killed for histological examination in groups, 24 hours, and 5, 15, 25, and 35 days after the inoculation.

Experiment 2.—Twenty-seven white mice were immunized with a subcutaneous injection of defibrinated blood as in Experiment 1. They were killed in groups 24 and 48 hours, and 4, 6, 8, and 10 days after the treatment and sections taken of the lymphoid organs.

Experiment 3.—Ten mice were immunized as in the previous experiments and 10 days later were inoculated with bits of Bashford Adenocarcinoma No. 63 as in Experiment 1. They were killed in groups and tissues taken at daily intervals from 24 hours to 4 days after the inoculation.

Experiment 4.—Twenty-eight normal mice were immunized as before. Four mice were killed for tissues 4 days afterwards, and on the 10th day after immunization four more were killed. The remaining twenty immunized mice were inoculated with bits of Bashford Tumor No. 63 10 days after the immunizing injection. They were killed in groups for tissues 2, 5, 15, 25, and 35 days after the inoculation.

Experiment 5.—Nine normal white mice were inoculated intraperitoneally with 0.6 cc. of defibrinated blood from normal mice. These were killed, three at a time, 24 hours, and 3 and 5 days after the inoculation, and the subcutaneous connective tissue was taken for examination.

All the mice used in the experiments were of about the same size and were from the same stock. The virulence of the tumors used in each experiment was tested by inoculation into a number of normal mice.

The fixative used for tissues was Conroy's 6-3-1, and the staining was done with Heidenhain's iron-hematoxylin for mitotic figures and eosin-methylene blue for other general purposes. In most instances loose connective tissue from the subcutaneous layer was carefully spread over the slide, fixed with absolute alcohol, and stained with methylene blue and eosin. Blood films were treated with Wright's stain.

OBSERVATIONS.

Much variation has been noted in the extent of the reaction in different animals. It has been shown by experiment that a certain percentage of mice are not immune after tissue injection but will grow tumors actively. In the earlier stages of the immunity reaction at which we have studied the lymphoid tissue it is unfortunately im-

possible to predict which of the animals would have fallen into the non-immune group. Therefore we are unable to say whether the animals failing to show the typical reaction of the majority would have proved to be non-immune if they had been allowed to live.

Spleen.—The stimulation of germinal centers was manifest 48 hours after the blood injection. In a section taken at this stage an average nodule usually contained a few well marked mitotic figures, three to five as a rule, more rarely six or seven. All stages of mitosis were easily distinguished. Among the cells of the germinal center were found a few large cells with pycnotic and fragmented nuclei. There was the usual number of pigmented cells and megalocaryocytes in the pulp spaces. The frequency of mitosis in the germinal center after 4 days was apparently greater than before (Fig. 1). There was also a slight increase in the number of pigmented cells and megalocaryocytes. After 5 days the number of mitotic figures was seen to decrease somewhat. The abundance of megalocaryocytes and pigmented cells at this time was about the same as before. There were small groups of pycnotic cells appearing in the pulp at about this period. Similar conditions seemed to continue until about 10 days, when the proliferative activity of germinal centers returned nearly to the normal level, but a few mitotic figures were seen at this period also. Moderate numbers of pycnotic cells were also found in the nodules.

The cytological condition just described was more strongly marked 24 to 48 hours after the cancer inoculation (Fig. 2). At this period there was a general enlargement of the splenic nodules and the latter contained numerous mitotic figures. The change was far more extensive than that occurring in the previous period. The number of pycnotic cells in the nodules was insignificant.

The enhanced cell division was conspicuous for about 1 week following the cancer inoculation, but after this period the normal rate was gradually resumed. Toward the later period large numbers of pycnotic cells were found in the nodules, but pigmented cells were always infrequent.

About 35 days after the inoculation the general histological appearance of the organ had returned approximately to normal.

Lymph Glands.—The cytological changes observed in the mesenteric and inguinal lymph glands were very similar to those found in the spleen. A distinct acceleration in the rate of cell division in the lymph glands was observed 24 hours after the immunizing injection. Dividing cells were numerous in the germinal centers of the nodules and were not infrequent even in the lymph cord. At 48 hours the number of dividing cells was more or less decreased, but there was considerable variation among individual mice. Slight stimulation was indicated by the unusual frequency of mitosis as late as 10 days after the treatment. No appreciable change was observed in the medulla.

The germinal center of the nodule became decidedly restimulated soon after the cancer inoculation, as evidenced by numerous mitotic figures, and this condition, in a less marked degree, lasted for a considerable length of time. About 35 days after the inoculation, or probably a little earlier, cell division in the nodule subsided to the normal rate.

No special change has been observed in any other part of the gland.

Circulating Lymphocytes.—Lymphocytes in the circulating blood of immunized animals were often seen in the process of amitosis, especially during the first several days after the inoculation of cancer. Amitosis was observed in large as well as small lymphocytes, but more frequently in the former. Cells in typical stages of amitosis were found among others showing irregularly shaped, lobulated nuclei.

The accepted interpretation of amitosis is that it is not a method of cell multiplication¹⁹ but a means of increasing the nuclear surface to meet intensified metabolic activities of the cell.²⁰ Since the lymphocytes are presumably in their active functioning stage at the period just indicated, this conception may, in the present state of our knowledge, be applied to the case under consideration. Even if two caryomeres produced by amitosis eventually separate into two apparent cells, we would not be warranted at present in considering the process evidence of genuine cell multiplication, especially in view of the scanty amount of cytoplasm possessed by the resulting cells.

¹⁹ Conklin, E. G., *Biol. Bull.*, 1917, xxxiii, 396.

²⁰ Nakahara, W., *J. Morphol.*, 1917-18, xxx, 483.

Subcutaneous Connective Tissue.—As early as 24 hours after the injection of the blood a well marked cellular reaction was seen in the connective tissue about the groups of red cells. The great majority of cells participating in this reaction were small lymphocytes. Other types of cells such as polymorphonuclear leucocytes and macrophages were rare. After about 4 days, when the injected blood became more generally distributed in the subcutaneous connective tissue, the local reaction became more diffuse. Now, besides the large number of lymphocytes, plasma cells in considerable number (Fig. 3) were present, and macrophages, which were few in earlier periods, became abundant. Owing to the wide distribution at this period of the injected red cells, it was found that an extensive area of the subcutaneous tissue showed numerous lymphocytes and other white cells. Soon the area infiltrated by red cells diminished, and there was a corresponding lessening of the lymphoid reaction. This cellular infiltration continued for about 8 days, after which time only slight traces of blood could be recognized.

The cellular reaction about the cancer graft in naturally resistant or artificially immunized animals bears a close similarity to the reaction as described above. As has been shown by many previous investigators, an extensive inflammatory reaction, which is lymphoid in nature, precedes the destruction of the cancer tissue, but the reaction around the graft subsides promptly after the latter becomes completely necrotic.

The cells which participate in this reaction are in both instances mainly lymphoid. Da Fano's¹¹ cellular analysis of the inflammatory reaction about the regressive cancer shows that the lymphocyte, plasma cell, and macrophage are characteristic of the reaction. Since these cells are usually absent in normal subcutaneous tissue of the mouse, much significance has been attached to their abundance about the neoplasm.

In addition to the local reaction about cancer tissue, Da Fano claims that the plasma cells which are absent in normal connective tissue of the mouse appear 48 hours after the injection of the blood for immunization and they become more numerous in the succeeding days until the 4th day, when small groups of the cells are seen in every section. While Da Fano's statements were confirmed in some of our

experiments, in others we failed to confirm them. We found no lymphoid proliferation in the loose connective tissue at a distance from the area infiltrated with red blood cells, and the loose connective tissue was found to be apparently normal, even when local lymphocytic infiltration was well pronounced about the injected blood cells or cancer graft (compare Figs. 3 and 4). That Da Fano has misinterpreted the local reaction about the mass of injected blood cells as a general reaction in immunity becomes clear when the immunizing dose is given intraperitoneally. In the latter case although the immunity is effective, no appreciable lymphoid proliferation in the loose connective tissue results.

Other Organs.—The thymus and thyroid glands, the liver, kidney, and bone marrow were studied, but no special changes were detected. In a few instances there was a rather unusually large number of mitotic figures in the cells of the thymus, and mitotic figures were often seen among the lymphoid cells about the vessels of the liver. But these active cells were either too few in number or too inconstant in occurrence to be more than mentioned in connection with the immunity reaction.

DISCUSSION.

The conclusions suggested by the work of Murphy and Morton regarding the rôle of the lymphocyte in immunity to transplanted cancer are not only in harmony with previous observations on the factor of resistance to heteroplastic tissue grafting and on cellular reactions about the cancer graft in animals with natural or induced immunity, but have sustained subsequent experimental tests as well. An objection which may be raised against the original work is that the evidence of lymphoid proliferation was based entirely upon the results of blood cell counts. Tyzzer,¹⁰ for instance, appreciating the wide range of variability in the white cell counts of the tail vein in normal mice, expresses the opinion that the increase in the number of lymphocytes in the blood of immune mice indicated by Murphy and Morton may probably be greatly exaggerated. The validity of this objection has now been set aside by the present cytological work, which shows that, accompanying the lymphocytosis, there is a corresponding enhancement of the rate of cell division in lymphoid centers.

In the same article Tyzzer points out, as of considerable significance, the fact that no change in the lymphocyte count of the mice is seen after the immunizing injection, while, as a matter of fact, "an animal so treated now possesses qualities which were previously absent, or in other words had become immune." In the light of the facts brought out in the present paper this point may simply mean that the newly formed lymphocytes do not get into circulation in sufficient number to change the general blood picture until the inoculation of cancer is made. It is now clear that an animal which has become potentially immune possesses hypertrophic lymphoid elements, although this fact is not reflected in the blood counts. However, the mechanism of the lymphoid reaction has become, as it were, sensitized so that a very small amount of tumor is sufficient to induce a relatively large blood lymphoid response.

Another finding of special interest is the similarity of the cellular reaction about the injected blood to that about the cancer graft in the immunized animal. The types of cells which participate in the reaction are in both instances primarily lymphoid, including large and small lymphocytes, plasma cells, and phagocytic cells. Moreover, the two reactions are to be regarded as of the same nature in that they no longer continue after the invading cells are dead. This point becomes more significant when taken together with the fact that the cells in the lymphoid centers respond in the same way to the blood injection as they do to the cancer inoculation, the only difference being one of degree. It seems probable, therefore, that the lymphoid cells of an untreated animal are sufficient in quantity and quality to be an efficient defensive mechanism against such cells as red blood cells, with no proliferative ability, and against such other tissues as have been used to produce immunity, such as spleen, embryonic tissue, skin, etc. As a result of the primary stimulus the capacity of the lymphoid tissue to react has become so enhanced that it is capable of a massive reaction when the cancer is inoculated.

The irregularity of the results described in our present experiments were to be expected. It is well known that a proportion of mice immunized to cancer show no more resistance than normal mice, while in still others the resistance to cancer growth is at first not evident, the defensive mechanism asserting itself sufficiently to over-



come the cancer only after a period of growth has occurred. Unfortunately at the time of the greatest changes in the spleen and lymph glands it is impossible to predict which way the animals would have arranged themselves according to this grouping. To take an example, ten mice are inoculated with cancer fragments 10 days after receiving an immunizing injection. If they are allowed to live the result would be as follows, judging by the average experiment with our strain of tumor: about two would develop tumors, about two more would show a temporary growth followed by retrogression, and the remaining six would be immune from the beginning. If all these animals are killed during the first few days after inoculation we have no way of comparing the extent of the reaction in the spleen with the degree of immunity which the animal might have shown if allowed to live. Therefore we would expect, if the changes in the spleen and lymph glands are an index to the immunity, only a proportion of the mice to show a marked reaction and from 10 to 20 per cent a delayed reaction. In our previous studies of the blood it was noted that the degree of immunity followed fairly closely the extent of the lymphocytosis.

SUMMARY AND CONCLUSIONS.

Mice immunized against cancer by means of an injection of defibrinated blood show in the germinal centers of the lymphoid organs a marked increase in the numbers of mitotic figures. The increase becomes evident 48 hours after the injection in the majority of instances and reaches its climax at about the 5th day. After this time it subsides, returning to the normal rate about the 10th day.

These immunized animals, when inoculated with a cancer graft 10 days after the injection, show a second stimulation of the lymphoid centers similar to the first but more intense in character. This increase in the number of mitotic figures becomes evident as early as 24 hours after the cancer inoculation and persists in a marked degree for a week, after which there is a gradual return to the normal rate.

The lymphocytes of the circulating blood during the establishment of the immunity show frequent examples of amitotic division, and many examples of irregular and lobulated nuclei. These changes suggest intensified functional activity.

Contrary to the statement of Da Fano, cellular reaction in the subcutaneous tissues of immunized animals is present only in the region infiltrated by the injected cells. This fact becomes conspicuous when the immunizing injection is given intraperitoneally, in which case no cellular accumulations are noted in the loose connective tissues.

No constant cellular changes were noted in the bone marrow, thymus or thyroid gland, liver, or kidney of the treated animals.

EXPLANATION OF PLATES.

PLATE 1.

FIG. 1. Germinal center of the spleen 4 days after the blood injection. *M*, mitotic figure.

FIG. 2. The same, 48 hours after the cancer inoculation in the immunized mouse. *M*, mitotic figure.

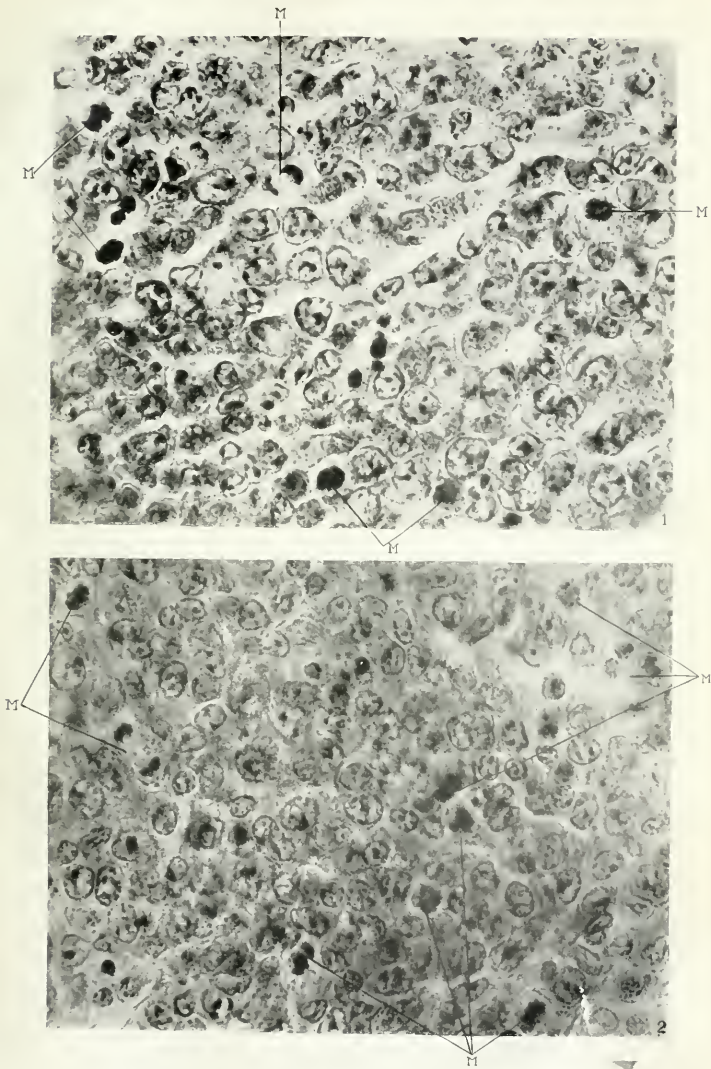
PLATE 2.

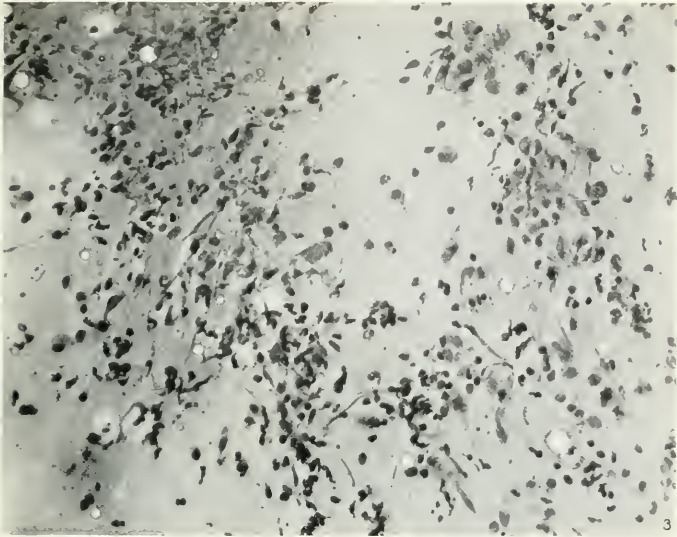
FIG. 3. Lymphoid infiltration in the subcutaneous tissue of an immunized mouse, the dark background indicating the presence of injected blood.

FIG. 4. A portion of subcutaneous tissue of an immunized mouse free from the injected blood, showing apparently normal cellular conditions.

(2)

W





(Murphy and Nakahara; Resistance to transplanted cancer. V.)

STUDIES ON X-RAY EFFECTS.

V. EFFECT OF SMALL DOSES OF X-RAYS OF LOW PENETRATION ON THE LYMPHOID TISSUE OF MICE.

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(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 3 TO 5.

(Received for publication, October 29, 1919.)

The destructive effect of x-rays on the lymphoid tissue was early noted in the study of the biological effects of this agent. The stimulating action of this agent on the circulating lymphocytes was first observed in this laboratory and was applied experimentally in the study of x-ray effects on spontaneous tumors of mice.¹ The earlier work was carried out with the old type of gas tube, with which it was difficult to regulate the amount and character of rays used, and therefore no attempt was made to standardize the dosage. This difficulty has largely been overcome by the use of the Coolidge tube. The stimulating dose for rabbits has been reported.² The work of Russ, Chambers, Scott, and Mottram³ confirms our earlier observation on the stimulative action in mice.

In our experiments with rabbits a histological study paralleling the blood counts confirmed the general nature of the stimulation by showing a marked increase in the number of mitotic figures in the germinal centers of the lymphoid organs of these animals.⁴ As mice are the animals used most extensively in our experiments, it was regarded as important to duplicate the histological study of the

¹ Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 800.

² Thomas, M. M., Taylor, H. D., and Witherbee, W. D., *J. Exp. Med.*, 1919, xxix, 75.

³ Russ, S., Chambers, H., Scott, G. M., and Mottram, J. C., *Lancet*, 1919, i, 692.

⁴ Nakahara, W., *J. Exp. Med.*, 1919, xxix, 83.

lymphoid organs of the latter animals after a stimulating dose of x-rays. With this end in view, the following experiments were undertaken.

Methods.

In the analysis of the artificial stimulation of the lymphocytes the reaction in the lymphoid germinal centers must be studied first. The spleens and lymph glands from the stimulated animals were fixed with Conroy's 6-3-1 fluid and stained with Heidenhain's iron-hematoxylin. The reaction was analyzed mainly from the standpoint of the frequency of mitosis.

EXPERIMENTS.

Experiment 1.—Seven normal white mice were placed in a glass jar and exposed to the following dose of x-rays: spark-gap $\frac{1}{8}$ inch between points, milliamperage 25, distance from the target to the back of the animal approximately 8 inches, time 20 minutes. The top of the jar was covered to shield the animal from the heat of the tube. The mice were killed 24 hours, and 3, 5, 7, 10, 12, and 14 days, respectively, after the treatment and the lymphoid organs examined histologically.

24 hours after treatment numerous degenerated cells, with pycnotic or fragmented nuclei appeared in the spleen (Fig. 1). The Malpighian bodies were small and inconspicuous. The general histological condition simulated somewhat the picture described by Heineke⁵ and Warthin⁶ after a massive dose of x-rays, but the changes were not so extensive. No appreciable amount of pigment was seen, but there was a moderate number of mitotic figures distributed irregularly. The necrotic changes were not so marked in the lymph glands as in the spleen, but mitotic figures were almost totally absent from the former.

3 days after treatment the necrotic cells were found to be decreased in number. The Malpighian bodies of the spleen were more or less evident but showed few mitotic figures.

After 5 days, and up to 14 days, the general histological condition of the lymphoid organs was about normal. There was no increase in the number of mitotic figures.

⁵ Heineke, H., *Mitt. Grenzgeb. Med. u. Chir.*, 1905, xiv, 21.

⁶ Warthin, A. S., *Physician and Surg.*, 1907, xxix, 1.

It must be concluded that no lymphoid stimulation occurred in the mice used in the preceding experiment. The dose of x-rays used was one which yielded evidences of lymphoid stimulation without appreciable destruction in rabbits.

Experiment 2.—Three normal white mice were treated in the same way and with the same dose of x-rays as the mice in Experiment 1, except that the time of exposure was reduced to 10 minutes. These animals were killed 24 hours, and 4 and 8 days, respectively, after the treatment and the lymphoid organs studied.

A considerable increase in the number of mitotic figures in the nodule of the spleen (Fig. 2) was seen at 24 hours and at 4 days after treatment with x-rays. The rough estimate of the average number of these figures in a section of one nodule of the spleen was five or six at these periods. This is in striking contrast with the normal condition, in which mitotic figures in a splenic nodule are few. No other special change was observed, except an appreciable increase of pycnotic cells in the pulp at 24 hours after x-rays. This, however, was not so conspicuous as in the previous experiment.

The lymph glands showed an equally pronounced increase in the mitotic figures at the corresponding period (Fig. 3), and figures were also seen frequently in the medulla.

8 days after the x-ray treatment the spleen and lymph glands, as regards the number of mitotic figures, were normal in appearance, but gave the impression of having more abundant lymphoid elements.

Experiment 3.—Five normal white mice were x-rayed as before. In this experiment, however, the time of exposure was reduced to 5 minutes, the other factors remaining unchanged. The mice were killed 24 hours, and 4, 7, 10, and 14 days, respectively, after the exposure.

Histological examination of the lymphoid organs showed no marked change, as regards the number of mitotic figures, throughout the periods studied. Moreover, the evidences of destruction which were so marked in the first experiment and much less so in the second one were not present.

From the three series of experiments described we may say that the indication is that stimulation of lymphoid tissue in the mouse may be effected by a 10 minute exposure to a small dose of x-rays. To

establish this point the following additional experiments were made, triplicating Experiment 2.

Experiment 4.—Three normal mice were exposed to x-rays of the same quality as in the previous experiments for 10 minutes. They were killed 24 hours, and 4 and 8 days, respectively, after the treatment.

Experiment 5.—Five normal mice were treated with the 10 minute dose as before and were killed 24 hours, and 3, 5, 7, and 10 days, respectively, after treatment.

In the mice killed at 24 hours and 4 days after treatment in Experiment 4 and in those killed 24 hours and 3 days after treatment in Experiment 5, an abnormally large number of mitotic figures was observed in the lymphoid centers, just as had been observed in Experiment 2. The other mice of this series showed no such change. The initial destruction was the same as that observed in Experiment 2.

DISCUSSION.

The uniformity of the changes, as shown in the three experiments, both in extent and period of occurrence, cannot be considered as a mere coincidence. It is to be concluded, therefore, that the small dose of x-rays employed in these experiments is capable of stimulating to proliferation the lymphoid tissue of mice.

It is of interest to note in this connection the apparent relation between the extent of cellular destruction and the degree of cellular stimulation, as shown in Table I. It would seem from these observations that a certain amount of destruction is followed by proliferation, which occurs very slightly, if at all, after too much or too little destruction. While no definite conclusion should be drawn on this point from our small series of experiments, they seem to be of sufficient interest to be reported.

TABLE I.

Time of exposure.	Destruction.	Stimulation.
<i>min.</i>		
20	+	±
10	±	++
5	—	—

From our present knowledge of the subject it is safe to say that the quantitative increase of the lymphoid elements in the body is mainly due to the hyperactivity of the lymphoblastic tissue of the lymphoid organs. Because of this activity hypertrophy of lymphoid organs, especially of the Malpighian bodies, and lymphocytosis in the blood may both result. Theoretically, as regards the number of these cells thrown into the circulation, it is conceivable that individual animals may react differently even with an equal stimulation of the lymphoid organs. Cases have been observed in which the blood lymphocytosis was due apparently to the mere emptying of the lymphoid organs without a corresponding actual increase of the lymphoid cells having taken place; and the histological studies just described have certainly given more nearly uniform evidence of stimulation than have blood counts.

CONCLUSION.

A dose of x-rays governed by the following factors induces a stimulation of lymphoid tissue in mice: spark-gap $\frac{7}{8}$ inch, milliamperage 25, distance 8 inches, time of exposure 10 minutes. Within 4 days after this dose there appeared an abnormally large number of mitotic figures in the lymphoid tissue of spleen and lymph glands, indicating an acceleration of the proliferative activity of the tissue.

EXPLANATION OF PLATES.

PLATE 3.

FIG. 1. A splenic nodule, 24 hours after a 20 minute exposure to the small dose of x-rays. Note the abundance of pycnotic cells.

PLATE 4.

FIG. 2. A splenic nodule, 24 hours after a 10 minute exposure to the small dose of x-rays. *M*, mitotic figure.

PLATE 5.

FIG. 3. A nodule of the mesenteric lymph gland, 4 days after a 10 minute exposure to the small dose of x-rays. *M*, mitotic figure.

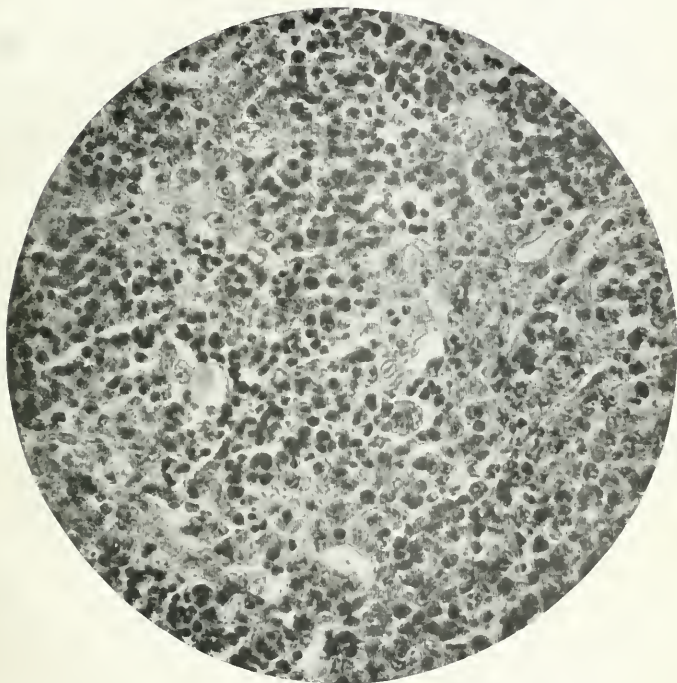


FIG. 1.

(Nakahara and Murphy: Studies on x-ray effects. V.)

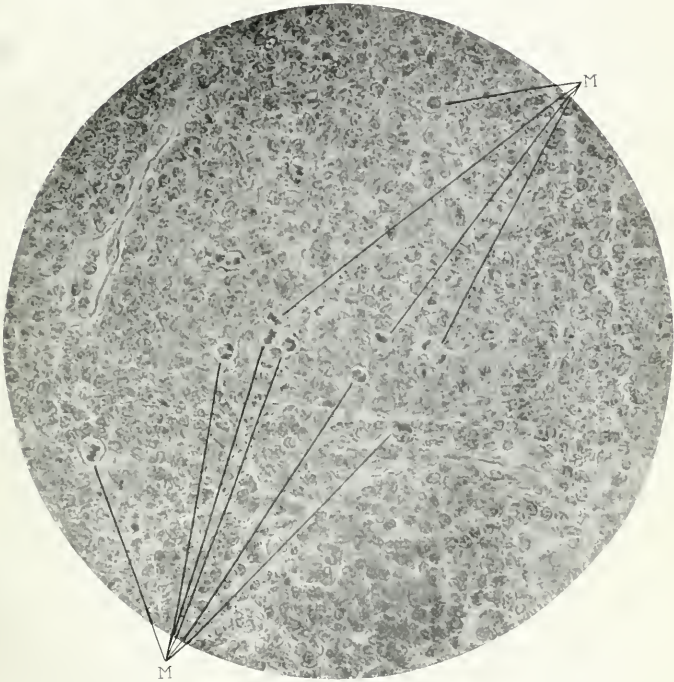


FIG. 2.

(Nakahara and Murphy; Studies on x-ray effects. V.)

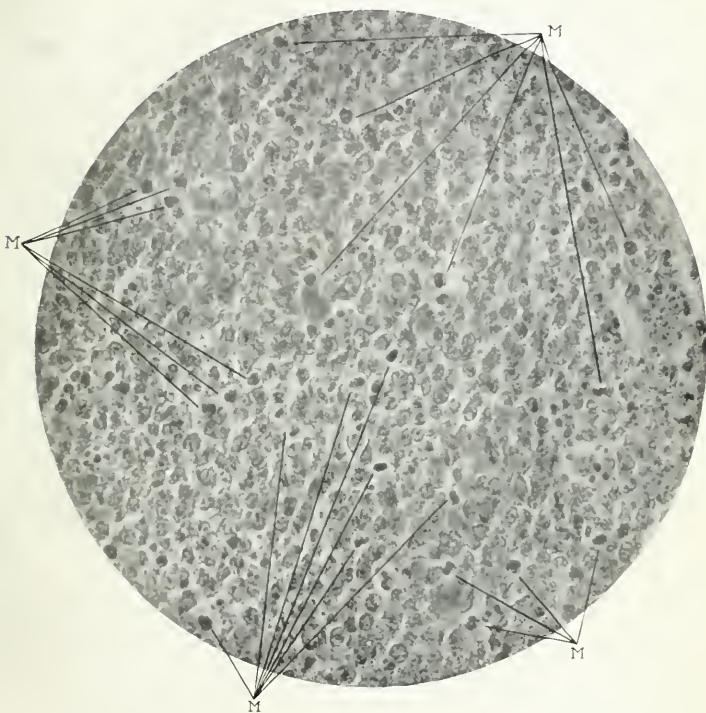


FIG. 3.

(Nakahara and Murphy; Studies on x-ray effects. V.)

TOXINS AND ANTITOXINS OF BACILLUS DYSENTERIÆ SHIGA.

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PLATES 6 TO 8.

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The nature of the toxin of the Shiga dysentery bacillus has been studied by a number of bacteriologists, notably by Shiga,¹ Neisser and Shiga,² Conradi,³ Vaillard and Dopter,⁴ Rosenthal,⁵ Todd,⁶ Kraus and Doerr,⁷ Flexner and Sweet,⁸ Doerr,⁹ Pfeiffer,¹⁰ Bessau,¹¹ and Lüdke.¹² But no agreement as to its precise nature has as yet been reached.

The chief discrepancies in experimental results and in deductions arrived at from them may perhaps be due to difference in method of preparing the toxin. Those who regarded the latter as an endotoxin prepared it by washing off the growth on agar slants with saline solution, shaking, heating to 56° or 60°C., incubating for 24 to 48 hours, and filtering through a Berkefeld candle. Those, on the other hand, who viewed it as an exotoxin obtained it by growing the bacilli in alkaline broth for a period of 2 to 6 weeks and then filtering.

¹ Shiga, K., *Centr. Bakteriöl., Ite Abt.*, 1898, xxiii, 599.

² Neisser, M., and Shiga, K., *Deutsch. med. Woch.*, 1903, xxix, 61.

³ Conradi, H., *Deutsch. med. Woch.*, 1903, xxix, 26.

⁴ Vaillard, L., and Dopter, C., *Ann. Inst. Pasteur*, 1903, xvii, 486.

⁵ Rosenthal, L., *Centr. Bakteriöl., Ite Abt., Ref.*, 1904, xxxiv, 503; *Deutsch. med. Woch.*, 1904, xxx, 235.

⁶ Todd, C., *Brit. Med. J.*, 1903, ii, 1456; *J. Hyg.*, 1904, iv, 480.

⁷ Kraus, R., and Doerr, R., *Wien. klin. Woch.*, 1905, xviii, 158, 1077.

⁸ Flexner, S., and Sweet, J. E., *J. Exp. Med.*, 1906, viii, 514.

⁹ Doerr, R., *Das Dysenterietoxin*, Jena, 1907, 30 ff.

¹⁰ Pfeiffer, *Centr. Bakteriöl., Ite Abt., Ref., Beilage*, 1908, xlii, 1.

¹¹ Bessau, G., *Centr. Bakteriöl., Ite Abt., Orig.*, 1910, lvii, 27.

¹² Lüdke, H., *Die Bazillenruhr*, Jena, 1911, 90.

Our studies of the toxic products yielded by the Shiga bacillus have led us to the conclusion that this microorganism growing *in vitro* produces two poisons, one an endotoxin, the other an exotoxin, which can be separated experimentally and can also be shown to attack different anatomical structures of the rabbit and to set up two distinct kinds of pathologic effects.

Shiga first pointed out that the bacillus which bears his name is highly toxic for the rabbit, and this animal has remained the chief one for demonstrating experimentally the pathogenic action of the microorganism. The Shiga bacillus or its poisonous products induces two kinds of marked lesions in the rabbit; one is localized in the intestine, and the other in the central nervous system.

The first comprehensive study of the lesions in the central nervous system was made by Dopter.¹³ He concluded that the central nervous system is usually the seat of serious lesions which may occur in any portion of the system, although the medulla is most often affected. The gray matter, and almost exclusively the anterior horns, show chromatolysis of the neurons in a varying degree and, besides, at times, areas of necrosis which destroy the cellular elements and myelin fibers, leaving scarcely any vestiges of them. At the same time there are an intense hyperemia and even hemorrhages invading the tissue. The white matter is intact. In short, the lesion is that of an acute myelitis, often an anterior poliomyelitis, and sometimes a polioencephalitis as well.

The intestinal lesions were studied by Flexner and Sweet⁸ who state that they vary in intensity. The coats of the large intestine are greatly thickened by inflammatory edema, in which case the mucosa is yellowish white and thrown into deep folds and corrugations, or more or less hemorrhage may be associated with the edema. At another time the transverse folds of mucous membrane are affected chiefly; they are swollen, the edges are hemorrhagic, and a pseudomembrane is scattered over the surface. Or, again, the transverse folds are greatly affected and the intervening mucosa is less affected, while patches of swollen and hemorrhagic mucous membrane, covered with a false membrane, appear upon and between the folds. The hemorrhage may extend into the serous coat.

The Exotoxin of Bacillus dysenteriae Shiga.

Preparation of the Exotoxin.—Comparative studies were made of media favoring a high yield of exotoxin as well as the conditions influencing its production. The following protocols are illustrative.

¹³ Dopter, C., Les dysenteries, Paris, 1909, 75 ff.; *Ann. Inst. Pasteur*, 1905, xix, 353.

*A. Yield of Toxin in Plain Broth and Egg Albumin Broth.*¹⁴—A quantity of plain meat infusion broth was divided into two lots. To the first was added one-third of its volume of a 10 per cent egg albumin solution. Both lots were adjusted to a pH of 7.8 and were inoculated with the same amount of a 24 hour broth culture of Shiga bacilli, Strain 114 S. After incubating for 5 days the cultures were filtered through a Berkefeld N candle and tested on rabbits weighing 1.500 gm. Table I shows the results.

TABLE I.
Yield of Toxin in Plain Broth and Egg Albumin Broth.

Medium.	Rabbit No.	Amount inoculated intravenously.	Results.
		cc.	
Plain broth.	1	1.0	Paralysis of posterior extremities in 24 hrs. Died in 36 hrs. No intestinal lesions.
" "	2	0.5	Weakness of anterior extremities in 3 days. Recovered. No intestinal symptoms.
Egg albumin broth.	3	1.0	Paralysis of anterior extremities in 18 hrs. Died in 36 hrs. No intestinal lesions.
" " "	4	0.5	Paralysis of both extremities in 36 hrs. Died in 48 hrs. No intestinal lesions.

B. Yield of Toxin in Media with Different Degrees of Aeration.—500 cc. of egg albumin broth were placed in a 2 liter flask giving the medium a surface diameter of 17 cm. and a depth of 2 cm. An equal amount was placed in a 500 cc. flask giving the medium a surface diameter of 7 cm. and a depth of 9 cm. Both lots were inoculated with Shiga bacilli, Strain 109, incubated for 7 days, filtered, and tested (Table II).

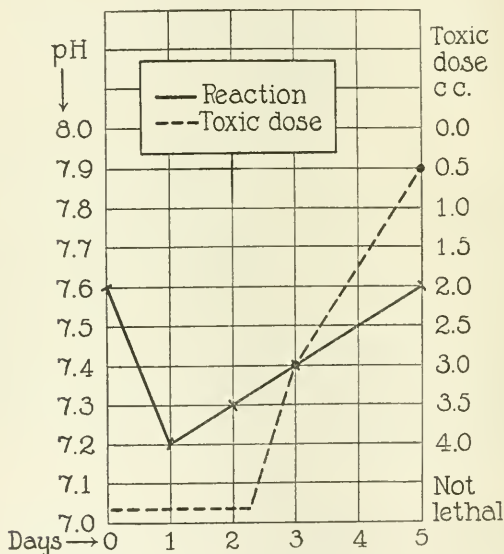
TABLE II.
Yield of Toxin in Media with Different Degrees of Aeration.

Condition of medium.	Rabbit No.	Amount inoculated intravenously.	Results.
		cc.	
Deep.	5	0.05	No effect.
"	6	0.10	Paralysis in 4 days.
"	7	0.50	" " 2 "
Shallow.	8	0.01	No effect.
"	9	0.05	Paralysis in 1 day.
"	10	0.10	" " 2 days.

¹⁴ It is to be understood where a single protocol only is given that all the experiments were repeated one or more times.

C. Relation of the Reaction of the Medium to the Yield of Toxin.—In order to determine the relation of the reaction of the medium to toxin production, small amounts of the culture fluid were tested daily with respect to reaction change and toxic potency.

Lot 15 of egg albumin broth, with an initial reaction of pH 7.6, was inoculated with Shiga bacilli and incubated at 37°C. The results are shown in Table III.



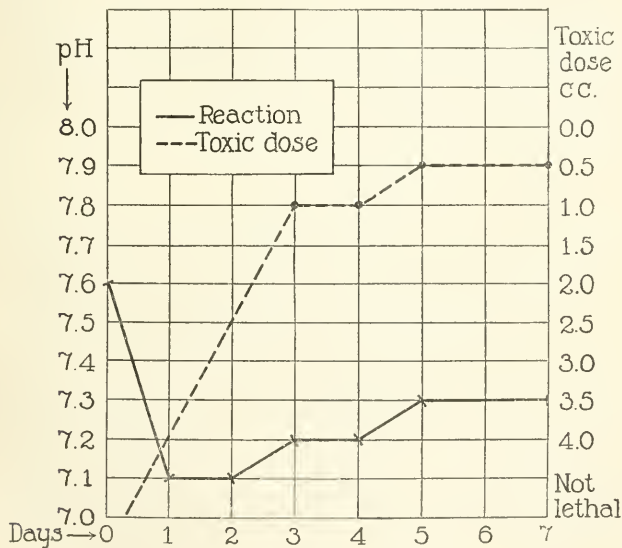
TEXT-FIG. 1. Relation of the pH of the medium to exotoxin production. Broth rendered sugar-free.

It is evident that the changes in the reaction of the medium, the development, in itself, of acid, do not influence the toxicity. But, as is shown in Text-figs. 1 and 2, no toxin is produced in the acid phase; it appears at the beginning of the alkaline phase, and increases thereafter.

TABLE III.

Relation of the Reaction of the Medium to the Yield of Toxin.

Length of incubation.	Reaction.	Toxicity.
<i>hrs.</i>	<i>pH</i>	
0	7.6	
24	7.2	None.
48	7.2	"
72	7.3	Minimum lethal dose, 5 cc.
96	7.4	
120	7.4	Minimum lethal dose, 1 cc.
7 days.	7.6	" " " 0.5 "



TEXT-FIG. 2. Relation of the pH of the medium to exotoxin production. Broth not rendered sugar-free.

D. Relation of the Muscle Sugar Content of the Medium to the Yield of Toxin.—Lot 14, egg albumin broth, was not rendered sugar-free but otherwise was treated like Lot 15. There was no striking difference in the toxicity, as revealed by comparing Text-figs. 1 and 2. The removal of the muscle sugar does not influence the toxicity.

On the basis of these experiments the procedure adopted for the production of exotoxin was as follows:

Plain meat infusion broth was mixed with one-third its volume of a 10 per cent solution of egg albumin. The latter was prepared by adding one volume of the whites of eggs to nine volumes of distilled water. The mixture was adjusted to pH = 7.6 to 7.8 and quantities of 500 cc. were distributed into 2 liter flasks to permit sufficient aeration and autoclaved for 45 minutes at a pressure of 15 pounds.

This medium was inoculated with one-half an agar slant of a 24 hour culture of the Shiga bacillus and incubated at 37°C. During the period of incubation the contents of the flasks were thoroughly shaken from time to time in order to increase aeration. At the end of 5 days the culture fluid was filtered through a Berkefeld N candle. The filtrate, if proved free from bacteria, constituted the exotoxin.

Nature of the Exotoxin.

Pathologic Effects.—The rabbit is very susceptible and reacts regularly to the action of the exotoxin, the effect depending on the amount injected.

A sublethal dose injected intravenously results in the development of paresis or paralysis of the extremities within 2 to 4 days. Both anterior and posterior extremities may be affected; the former are more frequently involved. The paralytic or paretic stage may endure for 1 to 3 days and may be followed by complete or partial recovery. During this period the animal is apathetic, has no appetite, and loses weight, but intestinal symptoms are either wholly absent or inconspicuous.

A lethal dose injected intravenously results in early paralysis and prostration; that is, within 24 to 48 hours. There is considerable loss of weight. Involuntary evacuations occur but without blood or mucus. Death follows in 1 to 2 days. The autopsy findings are illustrated in the following protocol.

Rabbit 11 (Figs. 1 to 3).—Oct. 29, 1917, 2 p.m. Injected intravenously with 0.05 cc. of Exotoxin 2. Oct. 31. Paralysis of posterior extremities. Temperature subnormal. Nov. 1. Prostrated. Incontinence of urine. Stools are frequent, formed, without blood or mucus. 3 p.m. Died.

Autopsy.—There were no evident lesions in the intestines (Fig. 3) or other viscera. The cerebrospinal system, however, showed severe lesions. The meninges were free from inflammatory reaction. The gray matter, especially of the medulla and cervical cord, and only slightly of the lumbar cord, was the site of the effects, which consisted of hemorrhage, quite extensive and visible to the naked eye, and, even oftener, multiple, discrete, interstitial hemorrhages as shown in Fig. 1. Areas of necrosis were scattered throughout the gray matter. A perivascular lesion was noted. The neurons showed atrophy or even complete dissolution in limited areas. In places there was chromatolysis, granular degeneration, or caryorrhexis of the cells. The perivascular lesion consisted in an infiltration of small round cells about the arterioles and capillaries, either as a single layer about or in the sheath of the vessels, or less frequently, as a dense, heaped up infiltration, as shown in Fig. 2. The same figure shows an excess of round cells throughout the gray matter. There was a moderate edema of the gray and white matter; otherwise the white matter and nerve fibers were not affected.

The lesions in the nerve tissue are characteristic and constant and agree with those described by Dopter¹³ of the nerve injury caused by the whole dysentery toxin, except that he did not describe the perivascular lesion.

The following series of experiments was undertaken to determine whether this toxin is of the nature of an exotoxin.

Period of Incubation.—To satisfy the requirements of the class of true toxins, a poison should show a definite period of incubation before the distinctive pathologic effects develop.

Experiment 1.—Oct. 8, 1917. Rabbit A injected intravenously with 0.05 cc. of Toxin 1. No effect. Rabbit B injected similarly with 0.1 cc. Oct. 9. Muscular weakness in both anterior extremities. Loss of 115 gm. in weight. Oct. 10. Recovered. Rabbit C injected similarly with 0.5 cc. Oct. 11 (3 days later). Muscular weakness of right anterior extremity. Oct. 12. Flaccid paralysis of this limb. Loss of 175 gm. in weight. Oct. 15. Paralysis improving. Oct. 17. Recovered. Rabbit D injected similarly with 1 cc. Beginning paralysis in 18 hours; complete paralysis of all limbs in 36 hours. Died in 60 hours. None of these rabbits showed intestinal involvement.

The incubation period therefore depends on the dose. The period for one minimum lethal dose is usually from 24 to 48 hours, although

we have noted from fifteen similar sets of experiments that it varies actually from a few hours to 4 days.

Globulin Fractionating.—Another point of comparison with the true toxins relates to the globulin fractionating of the poison.

Experiment 2.—Jan. 2, 1918. Rabbit A was injected intravenously with a globulin precipitate of Toxin 4. The globulin was purified and 0.2 cc. of a suspension in saline solution, equivalent to four minimum lethal doses, was injected. Jan. 3. Paralysis of both anterior extremities. Prostration. Loss of 70 gm. in weight. Jan. 4. Died.

Autopsy.—No visceral lesions. Macroscopic hemorrhages in gray matter of medulla.

Resistance to Heat.—An important difference between exotoxin and endotoxin is the thermolability of the former and the thermostability of the latter.

Experiment 3.—Several sets of rabbits were injected intravenously with four to ten minimum lethal doses of exotoxin which was heated for varying periods of time at temperatures from 60–90°C. As a control, endotoxin, to be described later, was submitted to similar tests. It was determined that the exotoxin was inactivated or destroyed when heated to 75°C. for 1 hour.

Production of Antitoxin.—The toxin yields an antitoxin which will be described in detail later.

The Law of Multiple Proportions.—The following experiment is selected from a series to show that Shiga exotoxin conforms to this law.

Experiment 4.—10 cc. of Toxin 4 equivalent to 100 minimum lethal doses were mixed with varying amounts of antiexotoxic serum and incubated for $\frac{1}{2}$ hour at 37°C. A series of rabbits was injected intravenously and it was found that 0.001 cc. neutralized one lethal unit. In other words, the antitoxic serum contained 1,000 antitoxic units.

Three other toxins were tested with the same antitoxic serum and all were neutralized in the same proportion.

Specificity of Neutralization.—A series of control experiments to determine the effect of non-specific sera on the exotoxin shows that no neutralization is obtained.

Experiment 5.—Three series of tests were made with normal horse, antitetanic, and antimeningococcic serum. Two to four minimum lethal doses of the exotoxin were mixed with 1 to 5 cc. of these sera and incubated for $\frac{1}{2}$ hour at 37°C. The

mixtures were then injected intravenously in rabbits. In all instances the typical neurotoxic effect of the toxin appeared.

Identity of Toxins of Different Strains.—From the standpoint of identification of the toxin as well as of specific therapy, it is desirable to know whether Shiga bacillus strains from different sources yield the same product. Exotoxins were prepared from the following strains: No. 100 from Newport News, Virginia, on artificial medium 1 year; No. 109 from Poughkeepsie, New York, 2 years; No. 114 F from Japan, more than 10 years; No. 114 S from Germany, more than 10

TABLE IV.
Rate of Production of Exotoxin.

Reaction of medium.	Period of incubation.	Amount of filtrate inoculated.	Results.	Classification.
pH	days	cc.		
7.6	0			
7.1	1			
7.1	2	5.0	No effect.	
7.2	3	1.0	Paralysis in 48 hrs. No intestinal lesions.	Exotoxin.
7.2	4	1.0	" " 48 " " " "	"
7.3	5	0.5	" " 48 " Died in 72 hrs.	"
7.3	7	0.5	" " 48 " " " 72 "	"
7.5	14	0.25	" " 48 " " " 72 " Intestinal lesions.	" + endotoxin.
7.8	21	0.25	No paralysis. Died in 48 hrs. Marked intestinal lesions.	Endotoxin.

years; and No. 114 T, source unknown, but on artificial medium for many years. The toxins yielded by all these strains were neutralized by the antitoxic serum produced with Strain 109.

It is evident then that strains from widely different sources produce similar exotoxins and that the exotoxin production is a constant phenomenon of the Shiga bacillus, modified slightly if at all by prolonged artificial cultivation.

Rate of Production.—The period of incubation of the culture has an important bearing on the production and nature of the toxic product. The prolongation of incubation leads, as will be shown later, to formation of endotoxin, which complicates the results. Table IV,

selected from a series of similar experiments, shows that the exotoxin develops relatively early and as incubation proceeds tends to diminish, while the endotoxin production rises.

To summarize, the Shiga bacillus grown in a favorable medium yields, in the first days of the cultivation, at the beginning of the alkaline phase of its growth, and before bacterial disintegration sets in, a toxic product which appears in the bacteria-free filtrate. This toxic product is precipitated with the globulin fraction of the protein, is relatively thermolabile, is capable of inciting antitoxin formation, is constant in properties, independently of the source of the Shiga culture, and produces in rabbits, after a definite incubation period, typical lesions of the central nervous system without at the same time, in an obvious way, injuring the intestines. In view of its peculiar properties we regard it as an exotoxin and a neurotoxin.

The Endotoxin of Bacillus dysenteriae Shiga.

Preparation of the Endotoxin.—The production of the endotoxin of Shiga bacilli does not differ essentially from that of other bacteria. The principle underlying all the methods is that of the autolysis or dissolution of the bacterial cell with the resultant liberation of its intracellular components. Most observations with the Shiga bacillus have been made with endotoxins produced in broth cultures by prolonged incubation (beyond 14 days). When a more rapid yield of endotoxin was desired, the following method was used.

Shiga bacilli were grown in Blake bottles for 24 hours. The growth was then washed off in saline solution, 15 cc. to each Blake bottle, incubated for 2 days at 37°C., and filtered through a Berkefeld N candle. We found that with Strain 100, 2.5 cc. of the filtrate prepared in this manner were lethal for rabbits weighing 1,500 to 1,800 gm.

Separation of Exotoxin from Endotoxin.—The technical difficulty of preparing pure endotoxin or exotoxin directly from the Shiga bacillus is great. Usually small amounts of one are found with the other. To establish the integrity of each of the two toxins and their independent action on the rabbit, separation of one from the other was necessary. The removal of exotoxin was accomplished by one of the methods given below.

Experiment 6. Separation by Heat.—Toxic Filtrate 16 was prepared by growing Shiga bacilli, Strain 100, in egg albumin broth for 22 days, and filtering.

Rabbit A (Control).—Injected intravenously with 1 cc. (four minimum lethal doses) of this filtrate. Paralysis of left posterior extremity after 48 hours, associated with a persistent blood-streaked mucous discharge from the intestines. Died after 4 days.

Autopsy.—Typical lesions in the medulla and intestines. Effects due to mixture of exotoxin and endotoxin.

Rabbit B.—The toxic filtrate was then heated at 80°C. for 1 hour. 1 cc. (four minimum lethal doses) was injected intravenously in Rabbit B. After 24 hours diarrhea but no nervous symptoms. Died after 4 days.

Autopsy.—Large intestine showed hemorrhagic and other lesions; cerebrospinal nervous system normal. Exotoxin destroyed by heat.

Rabbit C.—The toxin was also heated to 90°C. for 1 hour. Rabbit C was injected intravenously with 1 cc. (four minimum lethal doses). No effect. Exotoxin and endotoxin both destroyed.

TABLE V.

Neutralization Experiments with Various Combinations.

Shiga toxin. Class.	Antiserum.	Result.
Exotoxin.	Antiexotoxic.	No effect.
“ + endotoxin.	“	Intestinal lesions.
“ + “	“ + antibacterial.	No effect.
“	Antibacterial (containing antiexotoxin).	“ “

Experiment 7. Separation by Neutralization.—Toxic Filtrate 18 was prepared by growing the Shiga bacilli, Strain 100, in two Blake bottles for 24 hours, washing off with a total of 30 cc. of salt solution, incubating at 37°C. for 2 days, and filtering.

Rabbit A (Control).—Injected intravenously with 2 cc. (four minimum lethal doses) of Filtrate 18. After 20 hours, paralysis and intestinal symptoms. Died in 24 hours.

Autopsy.—Nervous and intestinal lesions. Mixture of exotoxin and endotoxin.

Rabbit B.—Injected intravenously with 2 cc. (four minimum lethal doses) of Filtrate 18 to which 1 cc. of antiexotoxic serum had been added previous to incubation for $\frac{1}{2}$ hour at 37°C. After 24 hours severe diarrhea and prostration. No nervous symptoms. Died after 3 days.

Autopsy.—Intestinal but no nerve tissue lesions. Neutralization of exotoxin by antiexotoxic serum.

Other combinations were tested by injection into rabbits as shown in Table V.

Nature of the Endotoxin.

Pathologic Effects.—Rabbits are as uniformly susceptible to the effects of the endotoxin as they are to those of the exotoxin. If a sublethal dose is injected intravenously the rabbit shows, after 24 to 48 hours, subnormal temperature, loss of weight, and diarrhea. The stools are frequent and mucoid, occasionally blood-tinged. This condition, during which no nervous symptoms are noted, endures for 2 to 3 days, after which the animal returns to normal.

If a larger but still sublethal, or a lethal dose is injected intravenously the animal reacts within 24 hours with subnormal temperature, considerable loss in weight, and prostration. Severe diarrhea arises, the stools being fluid and containing much mucus and more or less blood. The sensory and motor functions appear normal. The state lasts for 1 to 3 days, after which gradual recovery takes place, or death follows.

At autopsy the peritoneum is dull, its blood vessels are injected, and the peritoneal cavity contains a serous fluid. The small intestines are usually unaffected except that the vessels in the serosa may be injected. Occasionally the ileum is involved in the same extensive way as the large intestine. The walls of the latter are greatly thickened, edematous, injected, and show small discrete hemorrhages. A glairy gelatinous material covers the serous coat. On opening the intestines the contents are found to consist of blood-tinged mucus. The villi are hyperemic; the mucosa is swollen and reveals discrete hemorrhages and small ulcerations. In some instances necrotic areas are seen, and in one instance an area 2.5 cm. wide encircling the cecum was gangrenous. Microscopically destruction of the glandular elements, as well as a superficial general necrosis, is noted (Fig. 4). There is a cellular exudation in the submucosa and considerable edema and degeneration of the muscular layers. In the main, these pathologic effects in the intestine agree with the description given by Flexner and Sweet⁸ and others of the intestinal lesions produced by the injection of the whole dysentery toxin.

There are no lesions in the cerebrospinal nervous system. Hence this poison can be regarded, in contradistinction to the exotoxin, as an enterotoxin.

Resistance to Heat.—A property common to endotoxins is heat stability. We have determined that Shiga endotoxin is destroyed when heated at 85–90°C. for 1 hour.

Neutralization by Antiserum.—Antitoxin serum fails to neutralize endotoxin. Endotoxin, however, is neutralized by an antibacterial serum prepared by actively immunizing horses with Shiga bacilli.

To summarize, the Shiga endotoxin is a definite toxin, probably of intracellular origin, conforming to the properties of the endotoxins as a class. It differs physically and biologically from the Shiga exotoxin. Moreover, the two are separable by various procedures.

The Antitoxins of Bacillus dysenteriae Shiga.

That the exotoxin is capable of yielding an antitoxin serum is shown by the following experiment.

Experiment 8. Horse A.—Nov. 12, 1917. Injected intravenously with 5 cc. of Toxin 2 (prepared from Strain 109 grown in egg albumin broth 5 days and filtered) mixed with 1 cc. of polyvalent antidysenteric serum, as described below. Nov. 13. Injected similarly with 5 cc. of toxin but only 0.5 cc. of serum. Nov. 14. Same amount of toxin; 0.1 cc. of serum. Thereafter pure exotoxin was injected, the next series being started 7 days later with 1 cc. The intervals of injection, the increase of dosage, etc., followed the method given by Flexner and Amoss.¹⁵ Jan. 26, 1918. Dose increased to 30 cc., or a total of 80 cc. for the 3 day period of immunization. The horse reacted severely to this amount. Following this, single injections were given at weekly intervals, starting with 20 cc. and increasing slowly to 50 cc. Jan. 28. Trial bleeding; no antitoxin content. Nov., 1918. Trial bleeding; serum showed 1,000 antitoxin units per cc. Feb. 10, 1919. Trial bleeding; serum showed 2,000 antitoxin units per cc. (The basis for computation was one minimum lethal dose.)

Fig. 5 illustrates the results, selected from a series, of one of the neutralizing experiments made with this antitoxin serum. As shown by Rabbit B, the exotoxin is removed from a mixture of endotoxin and exotoxin by neutralization with the antitoxin serum; but as the endotoxin is unaffected the animal succumbed later and showed the intestinal lesions but no changes in the nervous system.

At this point a study was made of the stock polyvalent antidysenteric serum prepared at The Rockefeller Institute. This serum is ob-

¹⁵ Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1915, xxi, 515.

tained from horses repeatedly injected with live cultures of Shiga and Flexner bacilli according to the method of Flexner and Amoss.¹⁵ The serum tested was obtained from horses under immunization for several years (one horse 2 years, another 5 years). It was found that although the serum was prepared by injecting the cultures, it contained at least 2,000 antiexotoxigenic units per cc. as well as antienterotoxigenic and other antibacterial¹⁶ antibodies.

SUMMARY.

With the methods which have been described we have separated an exotoxin and an endotoxin from cultures of the Shiga dysenteric bacillus. The study of the nature and effect of the poison of this microorganism is thus simplified. The two toxins are physically and biologically distinct. The exotoxin is relatively heat-labile, arises in the early period of growth, and yields an antiexotoxigenic immune serum. The endotoxin, on the other hand, is heat-stable, is formed in the later period of growth, and is not neutralized by the antiexotoxigenic serum. The exotoxin exhibits a specific affinity for the central nervous organs in the rabbit, giving rise to a characteristic lesion—mainly, hemorrhages, necroses, and possibly a perivascular infiltration in the gray matter of the upper spinal cord and medulla. The endotoxin exerts a typical action on the intestinal tract, producing edema, hemorrhages, necroses, and ulcerations, especially in the large intestine.

In dysentery in man the intestinal lesions predominate, but in severe epidemics paralysis and neuritis have been observed (Osler¹⁷).

These facts become especially significant from the standpoint of the serum therapy of bacillary dysentery. A potent antidysenteric serum should contain antibodies against the exotoxin as well as the endotoxin. That such a serum can be produced in horses has been experimentally demonstrated.

¹⁶ Four lethal doses of endotoxin were neutralized by 0.01 cc. of this serum; on the basis of one lethal unit the serum may be said to contain 400 antiendotoxigenic units. The antibacterial antibodies tested were agglutinins. For some strains of Shiga bacilli the titer reached 1:20,000. In no instance was it less than 1:2,000.

¹⁷ Osler, W., *The principles and practice of medicine*, New York, 1912, 8th edition, 128.

EXPLANATION OF PLATES.

PLATE 6.

FIG. 1. Upper cervical region of the spinal cord of a rabbit injected intravenously with Shiga exotoxin. The hemorrhagic lesions in the gray matter, the edema, and the degeneration of the neurons are shown. $\times 85$.

FIG. 2. Section of the medulla of a rabbit injected intravenously with Shiga exotoxin. The perivascular lesion is shown. Three capillaries, indicated by arrow-heads, are seen in different stages of round cell infiltration. $\times 320$.

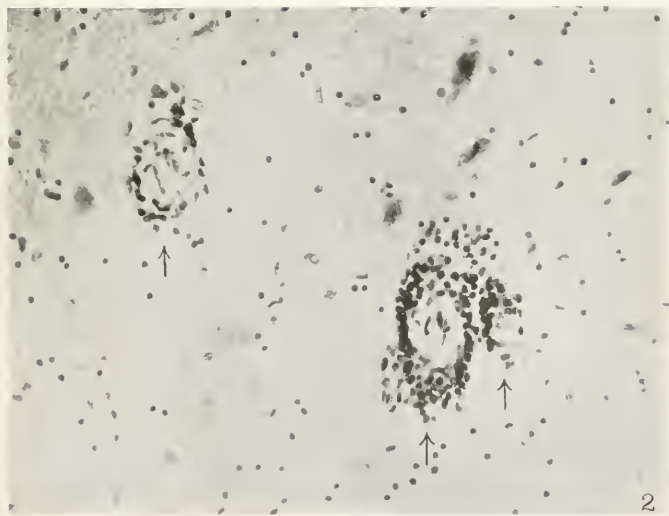
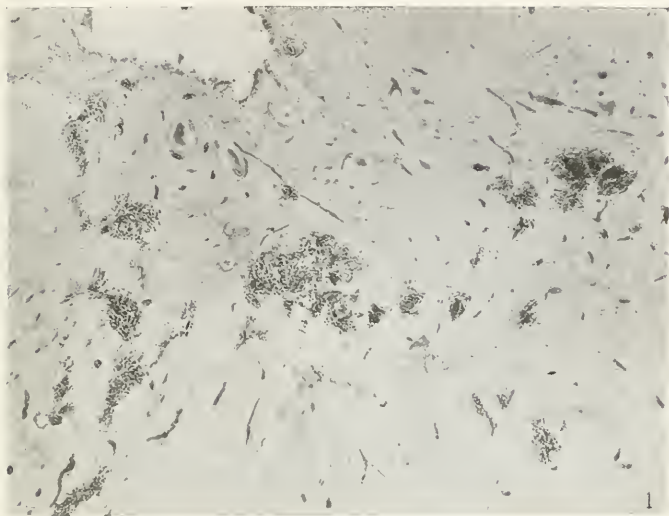
PLATE 7.

FIG. 3. Intestinal villus of a rabbit injected intravenously with Shiga exotoxin. The villus is not affected. $\times 72$.

FIG. 4. Intestinal villus of a rabbit injected intravenously with Shiga endotoxin. The superficial necrosis of the entire villus is evident. Most of the glandular elements are destroyed and the villus is considerably atrophied. $\times 72$.

PLATE 8.

FIG. 5. Rabbits A and B injected intravenously with filtrates containing both exotoxin and endotoxin. Rabbit A, injected with the mixtures of toxins incubated with normal horse serum, shows the effect of unneutralized exotoxin (paralysis and no intestinal symptoms). Rabbit B, injected with the mixtures of toxins incubated with antitoxin serum, shows the effect of neutralized exotoxin and unneutralized endotoxin (no paralysis but pronounced intestinal symptoms).



(Olitsky and Kligler: *Bacillus dysenteriae* Shiga.)



(Olitsky and Kligler: *Bacillus dysenteriae* Shiga.)



(Olitsky and Kligler: *Bacillus dysenteriae* Shiga.)

THE CULTURAL DIFFERENTIATION OF BETA HEMOLYTIC STREPTOCOCCI OF HUMAN AND BOVINE ORIGIN.

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Hemolytic streptococci are common in good dairy products and are usually harmless to the consumer. It is desirable to be able to distinguish such streptococci from hemolytic streptococci pathogenic to man which are at times found in dairy products. Up to the present there has been described no qualitative method for distinguishing hemolytic streptococci of human and bovine origin. Our ability to differentiate them depends upon the recognition of certain quantitative differences such as the agglutination or precipitation titer against an immune serum, the rapidity with which hemolysis appears, the size or definiteness of the hemolyzed zone in a blood agar plate, the rate of coagulation of milk, the degree of acidity produced in carbohydrate media, and the action of a bouillon culture on blood corpuscles in suspension. Anything which tends to make the determination of these quantitative differences more evident and simple is of value. Acquaintance with the minute details of appearances in blood agar is useful (1). Avery and Cullen (2) have shown the advantage of the determination of hydrogen ion concentration in dextrose bouillon cultures. We wish to emphasize the differential value of the action of streptococci of human and bovine origin on blood corpuscles in fluid media. The literature on the subject has been reviewed in a previous article.¹

Action of Hemolytic Streptococci on Blood in Fluid Media.

The Medical Department of the United States Army in 1918 recommended the following method for the identification of *Streptococcus hemolyticus* (3).

¹ Brown (1), Table IV.

"3. 0.5 cc. of the bouillon culture should be mixed with 0.5 cc. of a 5% suspension of washed rabbit blood corpuscles in physiological salt solution and incubated in a water bath at 37°C. for 2 hours. Markedly hemolytic pathogenic streptococci of human origin produce laking of blood under these conditions."

In Table I is recorded the result of the application of the above test to twenty-eight strains of streptococci of bovine and human origin. The strains tabulated were selected as representative of a large number of hemolytic bovine and human streptococci encountered by the author.

Since 1915 I have employed a slightly different technique from that described above for the same purpose and with practically the same results. A fresh young (18 to 20 hours old) standard veal or beef infusion bouillon culture is diluted to twenty times its volume with sterile 0.85 per cent salt solution. To 1 cc. of the diluted culture in small tubes in a Wassermann bath is added one drop of sterile defibrinated blood. The Wassermann tubes should be clean but need not be sterile. 2 hours incubation does not give opportunity for the growth of contaminating organisms to interfere with the reaction. The result of the application of this technique to the same streptococci as employed above is also shown in Table I.

The technique is perhaps a little simpler than that employed by the Army. It must not be assumed, however, that all mixtures of blood or blood corpuscles and bouillon culture will give similar results. It is a purely quantitative test and if it is to be of the maximum differential value the proportions described above must be adhered to. If the culture is diluted 1:10 some of the bovine strains produce hemolysis within the 2 hour period. If it is diluted 1:40 some of the human strains fail to produce hemolysis. Bouillon cannot be used instead of salt solution for making the dilutions. If more than one drop of blood is added the results are irregular. The bouillon cultures must be young and active. Experiments with centrifuged organisms indicate that the number of organisms present is of relatively little importance. The desired result attained by dilution of the culture appears to be due to dilution of the medium rather than dilution of the bacterial suspension. It is necessary to use a greater dilution of the culture when defibrinated blood is employed rather than washed corpuscles because the serum of the former serves as

TABLE I.

Action of Streptococci on Blood in Salt Solution and Bouillon.

Strain.	Source.	Hemolysis in 2 hrs. at 37°C.				
		Washed rabbit corpuscles, U. S. Army technique.	Defibrinated blood. Author's technique.			
			Horse.	Human.	Rabbit.	Beef.
A-Cow 1	Milk from individual cow.	—	—	—		
A-Cow 8	" " " "	—	—	—		
A-Cow 18	" " " "	—	—	—	—	—
A-Cow 25	" " " "	—	—	—		
B-Cow 18	" " " "	—	—	—	—	—
H-Cow 71	" " " "	—	—	—	—	—
H-Cow 72	" " " "	+	—	—	—	—
J-C10	" " " "	—	—	—		
J-C59	" " " "	—	—	—		
	(mastitis).					
J-MJ	Milk pail.	—	—	—		
J-E7	" from individual cow.	—	—	—		
J-C65	Udder of cow at autopsy (mastitis).	—	+	±		
Cheese 1	Cream cheese.	—	—	—	—	—
" 2	" "	—	—	—	±	—
B-Cow 2b	Milk from individual cow (mastitis).	++++	++++	++		
H-Cow 108	Milk from individual cow (mastitis).	++++	++++	+++	++++	+++
H-M	Human throat.	+++	+++			
X-32	" "	++++	+++	++	+++	+++
X-38	" lymph node.	++++	+++	++	+++	±
X-40	" spleen.	++++	++++	+++	++++	+++
X-43	" kidney.	+++	++++	++++		
Imp. 1	Impetigo contagiosa.	+++	++++	±		
" 2	" "	++++	++++	+++		
D-AD4	Human throat.	++++	++++	++++		
X-44	" empyema.	++++	++++	++		
X-45	" "	++++	++++	++++	++++	++++
X-46	" "	++++	++++	++++	++++	++++
A-SH	" peritoneal cavity.	++++	++++	++++		
Control.	Sterile salt solution.	—	—	—	—	—

Complete hemolysis is indicated by + + + +. Tubes were shaken each half hour.

medium for the streptococci. The importance of proper dilution and the influence of the length of period of incubation are shown in Table II. This table also illustrates strikingly the difference in hemolytic activity of bovine and human strains under these conditions.

[illegible]

The tubes were shaken after each reading to keep the blood corpuscles from settling out.

TABLE II—*Concluded.*

Strain and dilution.	Incubation at 37°C.				Strain and dilution.	Incubation at 37°C.				In room over night.
	½ hr.	1 hr.	1½ hrs.	2 hrs.		½ hr.	1 hr.	1½ hrs.	2 hrs.	
J-C59					Imp. 2					
1:5	—	—	—	±	1:5	++	++	++	++	++
1:10	—	—	—	—	1:10	++	++	++	++	++
1:20	—	—	—	—	1:20	—	++	++	++	++
1:40	—	—	—	—	1:40	—	±	++	++	++
1:80	—	—	—	—	1:80	—	—	++	++	++
J-MJ					D-AD4					
1:5	—	—	±	+++	1:5	++	++	++	++	++
1:10	—	—	—	++	1:10	++	++	++	++	++
1:20	—	—	—	—	1:20	++	++	++	++	++
1:40	—	—	—	—	1:40	—	++	++	++	++
1:80	—	—	—	—	1:80	—	—	++	++	++
J-E7					X44					
1:5	—	—	—	—	1:5	++	++	++	++	++
1:10	—	—	—	—	1:10	±	++	++	++	++
1:20	—	—	—	—	1:20	—	+	++	++	++
1:40	—	—	—	—	1:40	—	—	++	++	++
1:80	—	—	—	—	1:80	—	—	++	++	++
J-C65					X45					
1:5	—	—	—	±	1:5	++	++	++	++	++
1:10	—	—	—	—	1:10	++	++	++	++	++
1:20	—	—	—	—	1:20	++	++	++	++	++
1:40	—	—	—	—	1:40	++	++	++	++	++
1:80	—	—	—	—	1:80	±	++	++	++	++

Appearance in the Blood Agar Plate.

In the blood agar plate all the strains selected belong to the beta type. They show certain quantitative differences, however, as indicated in Table III.

TABLE III.
Hemolysis in Horse Blood Agar.

Strain.	Character of the zone produced by the deep colony.
A-Cow 1	A small clear sharply defined central zone and a broad outer partly hemolyzed zone.
A-Cow 8	
A-Cow 18*	
A-Cow 25	
B-Cow 18*	The zone is smaller and slower to develop than that of known human strains.
H-Cow 71	
H-Cow 72	
J-C10	
J-C59	
J-MJ	
J-E7	The zone is a little slower to develop than that of human strains but might be taken for the latter.
J-C65	
Cheese 1	
" 2	
B-Cow 2b*	Typical human strains. Clear colorless well defined completely hemolyzed zone 2-2.5 mm. in diameter after incubation for 18-24 hrs. at 37°C.
H-Cow 108	
H-M	
X-32	
X-38*	
X-40	
X-43	
Imp. 1	
" 2	
D-AD4*	
X-44	
X-45	
X-46	
A-SH	

* Blood agar plates of these strains are illustrated in a previous article (1).

The zones of hemolysis produced by the pathogenic beta type hemolytic streptococci of human origin are of uniform size and character, but those produced by the bovine strains show considerable variety. It is, therefore, often possible on seeing a deep colony in

a blood agar plate for the first time to state with a fair degree of certainty that the organism is not of the human pathogenic variety. However, since some bovine streptococci produce zones much like those of human strains, it is not possible to state positively that such a colony is of human origin. Four such strains are indicated in Table III.

Fermentation Reactions.

In Table IV is given the titratable acidity after incubation for 1 week of cultures in fermented bouillon plus 5 per cent of sterile horse serum and 1 per cent of the test substance indicated.

TABLE IV.
Fermentation Reactions.

Strain.	Titratable acid (per cent normal).					
	Saccharose.	Lactose.	Salicin.	Raffinose.	Inulin.	Mannite.
A-Cow 1.....	4.65	4.4	1.4	1.15	1.15	0.95
A-Cow 8.....	4.8	4.85	1.45	1.1	1.15	1.0
A-Cow 18.....	4.7	5.0	1.48	1.3	1.0	1.0
A-Cow 25.....	4.75	5.0	1.15	1.1	1.1	1.1
B-Cow 18.....	3.85	5.0	1.2	0.95	0.95	0.9
H-Cow 71.....	4.9	5.0	3.3	0.55	0.55	0.65
H-Cow 72.....	4.95	4.85	3.25	0.55	0.7	0.5
J-C10.....	5.3	4.8	4.4	0.6	0.55	0.3
J-C59.....	4.95	4.7	3.8	0.65	0.6	0.45
J-MJ.....	3.45	3.35	0.5	0.45	0.5	0.55
J-E7.....	4.75	4.6	3.7	0.5	0.55	0.5
J-C65.....	4.45	3.15	3.25	0.7	0.2	0.3
Cheese 1.....	5.7	4.7	5.0	0.8	0.35	3.8
“ 2.....	3.4	4.45	5.6	0.8	0.3	3.65
B-Cow 2b.....	3.6	3.65	4.8	1.05	0.9	0.85
H-Cow 108.....	3.6	3.2	3.2	1.0	1.0	0.8
H-M.....	3.55	3.7	3.55	0.8	0.6	0.85
X-32.....	3.55	3.55	4.7	0.9	0.75	0.95
X-38.....	3.95	3.65	4.8	0.8	0.85	0.8
X-40.....	3.85	3.15	4.9	0.8	0.8	0.7
X-43.....	3.9	3.25	3.4	1.05	1.0	1.0
Imp. 1.....	4.0	3.4	3.55	0.55	0.9	0.7
“ 2.....	2.9	3.5	2.95	0.6	0.5	0.35
D-AD4.....	3.25	2.85	2.65	0.85	0.85	0.7
X-44.....	3.7	3.45	3.35	0.4	0.75	0.7
X-45.....	3.9	2.8	2.5	0.35	0.25	0.3
X-46.....	3.6	3.75	3.8	0.35	0.1	1.5
A-SH.....	3.65	1.0	4.35	1.0	1.2	3.4

The titratable acidity of the medium was 0.5 to 1 per cent normal. A titratable acidity of less than 1.5 is regarded as a negative fermentation reaction.

Limiting Hydrogen Ion Concentration.

In Table V is given the hydrogen ion concentration of cultures in 1 per cent dextrose bouillon after incubation for 68 hours.

TABLE V.

Limiting Hydrogen Ion Concentration in Dextrose Bouillon.

Strain.	pH				Strain.	pH 68 hrs.
	24 hrs.	68 hrs.	72 hrs.	116 hrs.		
A-Cow 1.....		4.6			B-Cow 2b.....	5.2
A-Cow 8.....		4.5			H-Cow 108.....	5.2
A-Cow 18.....		4.6			H-M.....	5.2
A-Cow 25.....		4.5			X-32.....	5.2
B-Cow 18.....		4.4			X-38.....	5.2
H-Cow 71.....		4.5			X-40.....	5.1
H-Cow 72.....		4.6			X-43.....	5.2
J-C10.....		4.6			Imp. 1.....	5.3
J-C59.....		4.5			" 2.....	5.3
J-MJ.....	5.8	5.3		5.3	D-AD4.....	5.3
J-E7.....	5.1	4.6	4.3		X-44.....	5.4
J-C65.....		5.1			X-45.....	5.2
Cheese 1.....		4.4			X-46.....	5.2
" 2.....		4.4			A-SH.....	5.2

Hydrogen ion determinations were made colorimetrically according to Clark and Lubs (Clark, W. M., and Lubs, H. A., *J. Bacteriol.*, 1917, ii, 1, 109, 191) with methyl red as an indicator.

The streptococci studied by Avery and Cullen (2) reached their limiting hydrogen ion concentration within 24 hours. This was also true of all the known human strains and most of the bovine strains studied by the author. However, a few of the latter did not; *e.g.*, Strain J-E7. Neither could Strain J-MJ be depended upon in this respect. It was also noted that some substances, *e.g.* salicin, were fermented more slowly than others. The limiting hydrogen ion concentration was reached later in media containing this substance.

Growth in Milk.

Reference to Table VI shows that no sharp distinction between bovine and human strains can be drawn from their ability to coagulate milk in test-tubes. After incubation for 5 or 6 days most of the

strains, human and bovine, had coagulated the milk. In 24 hours only about half the bovine strains had done so. In 48 hours some of the human strains had partially coagulated the milk and some of the bovine strains had done no better. The most that can be said is that none of the strains of human origin caused coagulation of milk within 24 hours, whereas about 50 per cent of the bovine strains did so.

TABLE VI.
Coagulation of Milk.

Strain.	Period of incubation.			Strain.	Period of incubation.		
	24 hrs.	48 hrs.	6 days.		24 hrs.	48 hrs.	6 days.
A-Cow 1.	++	+++++	+++++	B-Cow 2b.	—	—	+++++
A-Cow 8.	++	+++++	+++++	H-Cow 108.	—	++	+++++
A-Cow 18.	+++	+++++	+++++	H-M.	—	++	+++++
A-Cow 25.	+++	+++++	+++++	X-32.	—	—	+++
B-Cow 18.	+++	+++++	+++++	X-38.	—	+	+++
H-Cow 71.	++	+++++	+++++	X-40.	—	—	±*
H-Cow 72.	+++	+++++	+++++	X-43.	—	—	+++
J-C10.	—	+	+++++	Imp. 1.	—	+	+++
J-C59.	—	+	+++++	" 2.	—	—	—*
J-MJ.	—	—	—*	D-AD4.	—	—	—*
J-E7.	+	++	+++++	X-44.	—	++	+++++
J-C65.	—	—	—*	X-45.	—	+	+++++
Cheese 1.	—	+	+++++	X-46.	—	+	+++++
" 2.	+++++	+++++	+++++	A-SH.	—	—	—†

* Coagulated when placed in boiling water.

† Not coagulated when placed in boiling water.

++++ indicates complete coagulation.

Reaction to Methylene Blue.

It has been claimed by Sherman and Albus (4) that cultures of *Streptococcus lacticus* reduce methylene blue in milk while those of *Streptococcus pyogenes* fail to do so and in fact fail to grow in this medium. Their results are striking, though we doubt the validity of their method of selecting cultures. We cannot agree in applying the name *Streptococcus pyogenes* to all streptococci isolated directly from the udder. The strains studied above were inoculated into methylene blue milk (1: 20,000) according to the technique described by Sherman

and Albus. The two strains from cheese reduced the methylene blue almost completely in less than 16 hours, but certain strains from the udder and from human patients also produced partial reduction in less than 24 hours, others within 66 hours, and others not at all. On methylene blue agar plates (1:20,000) streaks of Strains Cheese 1 and Cheese 2 grew luxuriantly, while none of the other strains grew at all. It is interesting to note, however, that streptococci which were inhibited by methylene blue in the presence of oxygen grew well in media containing methylene blue which was reduced by some other agent. This was demonstrated by boiling tubes of methylene blue agar in a water bath until the dye was colorless, inoculating the agar in fluid condition, chilling it quickly, and incubating. After several hours the blue color returned to the upper half inch of the medium and in this portion no colonies developed. Below the upper half inch the agar remained colorless and here the streptococcus colonies grew as well as in agar containing no methylene blue.

CONCLUSION.

None of the procedures described serves by itself to differentiate streptococci of human and bovine origin with certainty, though each of them serves as a strong presumptive test. Most strains fall easily into the human or bovine group by all the tests. Eliminating these from consideration we have left certain irregular strains listed in Table VII.

TABLE VII.
Irregular Strains.

Strain.	Source.	Origin as indicated by.		
		Blood agar plate.	Blood-salt solution.	Acidity in dextrose bouillon.
J-E7	Normal udder.	Human (doubtful).	Bovine.	Bovine.
Cheese 1	Cream cheese.	" "	"	"
" 2	" "	" "	"	"
J-C65	Diseased udder.	" "	"	Human.
J-MJ	Milk pail.	Bovine.	"	"

Taking all characters into consideration we are inclined to regard Strains J-E7, Cheese 1, and Cheese 2 as undoubtedly of bovine origin. Strain J-MJ also is representative of a group of streptococci which Jones² has found in milk and which is being further studied by him. There remains Strain J-C65 which for the present must be regarded as of doubtful origin.

The author is indebted to Dr. F. S. Jones for the strains of streptococci lettered J, and to Miss Marion L. Orcutt for valuable assistance.

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² Jones, F. S., personal communication.

DAIRY INFECTION WITH STREPTOCOCCUS EPIDEMICUS.

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PLATE 9.

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The milk-borne streptococcus epidemic about to be reported is of interest not because of any unusual features or because of any intensity of the epidemic, but because of the definiteness of the findings and the promptness with which they were obtained. There were also certain unusual clinical features.

The dairy involved was one producing a high grade of raw milk for sale in Boston. In February, 1917, there was prevalent, especially among children, a malady which was usually diagnosed as epidemic adenitis, more rarely as sore throat, pharyngitis, or tonsillitis. The initial symptom was sore throat of moderate severity, rarely such as to be diagnosed as septic sore throat, a reportable disease in Massachusetts. A few patients experienced a peculiarly nauseating nasopharyngitis. A few cases of otitis media occurred, but the most constant development was inflammation of the submaxillary and cervical lymph nodes. Recovery was sometimes quite slow and characterized by recurrent periods of high temperature.

At the time the authors were consulted by the dairy several pediatricians had noticed that among their patients an undue proportion were consumers of this milk. On the other hand, an infants' hospital supplied with milk by this dairy had reported no cases of the disease.

On the afternoon of February 23, a conference was called by the officials of the dairy. Physicians whose patients were using this milk were invited to attend this conference and also the authors. Although there was no conclusive evidence that the milk was at fault, a complete investigation was undertaken at once. The following measures were adopted. (1) At the conference physicians were sup-

plied with sterile swabs for the collection of pathologic material from patients. (2) Arrangements were made to obtain samples of milk from individual cows of the dairy herd beginning the next morning. (3) Swabs from the throats of employees of the dairy were to be examined as soon as the milk had been studied. (4) All the milk of the dairy was to be pasteurized in the final bottles for the time being.

Apparently the epidemic among consumers of the milk ended immediately. The initial symptoms of the last case reported were noticed on February 24.

Since in a paper by Dr. Theobald Smith and the author (1) the milk-borne epidemics described were designated A to G inclusive, this one and the dairy involved will be referred to as Epidemic H and Dairy H.

The Human Material.

Swabs received were soaked for 15 minutes in about 1 cc. of sterile salt solution which was pipetted directly into the tube in which the swab was received. The salt solution was then diluted, added to fluid blood agar (standard meat infusion agar, 12 cc., plus defibrinated horse blood, $\frac{2}{3}$ to 1 cc., at 45°C.), and immediately poured into a Petri dish. After the moisture condensing inside the dish had been allowed to evaporate the plate was incubated at 37°C. in the inverted position.

Fifteen swabs were received from patients using milk of Dairy H. Of these, twelve were from throats or tonsils and three from ears. In the blood agar plates of twelve of these swabs, including those from ears, was found the organism which we have learned to recognize as *Streptococcus epidemicus* Davis.¹ The appearance of this organism in the blood agar plate is such as to make it readily recognizable (2). It produces the beta type of hemolysis. The surface colonies are large and watery, round when isolated but confluent and amebiform in the streak. Organisms in the fresh surface colonies are encapsulated. This streptococcus was present in large numbers on most of

¹ The name *Streptococcus epidemicus* is used tentatively. The organism is so nearly like *Streptococcus pyogenes* that it may be regarded as a well defined variety of the latter rather than as a true species, and if so should be called *Streptococcus pyogenes* (var. *epidemicus*).

the swabs; in pure culture on three of them, two from throats and one from an ear, and in almost pure culture on several others. It was not found on three of the swabs from patients using Dairy H milk. One of these was a convalescent patient and the material from the other two was not properly diluted and the plates were poor. These should have been done over again, but in view of the evidence already at hand and because of the great quantity of material from cows waiting for study at the time, this was not done.

Swabs were studied from four adults in families where children had the infection. The children in these families used milk from Dairy H but the adults did not use it regularly. A diagnosis of epidemic adenitis was not made in the case of the adults but they complained of coughs, colds, or sore throat. In none of them was *Streptococcus epidemicus* found.

Nine swabs were studied from patients not using milk from Dairy H. Three of these were from cases with a clinical diagnosis of epidemic adenitis. The others were diagnosed as sore throat or tonsillitis. From none was *Streptococcus epidemicus* isolated. Other pathogenic streptococci were recovered from some of them.

Most of the patients from whom *Streptococcus epidemicus* was isolated had developed initial symptoms on February 13 to 18. Most of the swabs were taken on February 24, 25, and 26. The last case reported developed initial symptoms on February 24, and the swab was taken on March 2.

The Dairy Herd.

All the milk sold by Dairy H was produced by its own herd, about 112 cows being milked at this time. The milk was produced and handled under exceptionally good conditions. Precautions were taken to exclude the milk from cows with garget. The herdsman drew the fore-milk from each teat onto a fine wire screen in order to detect the presence of flocculi or viscosity. The so called gang system of milking was in use, each man milking the next cow in line at the head of the gang of milkers as he finished milking the last one. The milk was quickly cooled and bottled at the farm.

The herd was housed in three barns, A, B, and C. On the morning of February 24 samples of milk were taken from twenty cows in Barn A. A stream of milk from each of the four quarters of every cow was milked directly into a sterile cream bottle. The samples were packed in ice and taken directly to the laboratory where they were centrifuged and the sediment was plated out in horse blood agar. A stained film of the sediment was also made. In making the blood agar plates it was found useful to employ both deep and surface streak inoculation of the same plate. The tube of blood agar was first inoculated in fluid condition, then after the plate had been poured and hardened it was streaked in three or four places. This gave an opportunity to observe both deep colonies and surface streaks on the same plate.

It happened that one of the first samples yielded in pure culture large numbers of *Streptococcus epidemicus* resembling in every way the organisms isolated from the patients (Fig. 1). The stained film of the milk sediment revealed large numbers of leucocytes and many short chains and pairs of round or flattened cocci. The film alone would have attracted suspicion but could not have been relied upon for a diagnosis. The milk of this cow (No. 108) had not yet roused the suspicion of the herdsman or milkers and was being used. As soon as this culture was discovered the cow was isolated on February 25, and on the morning of February 26 samples of milk were taken from each quarter of the udder. By this time the sample from the left fore quarter was noticeably thick and yellow, while samples from the other three quarters were normal in appearance. Cultures showed the left fore quarter only to be infected.

By February 26 samples of milk from all the cows of the herd had been similarly cultured and examined. None revealed any streptococci resembling those from the patients and Cow 108. Among these cows, however, were three known to have garget in one quarter of the udder. The milk of this quarter was regularly discarded. We studied samples of milk from these quarters culturally and found streptococci which produced more or less hemolysis in the blood agar plate but did not closely resemble the streptococci isolated from the patients and Cow 108.

The location of Barn A and the method of handling the milk at the farm offer a suggestion as to why the epidemic was not more widespread among the patrons of the dairy. They also serve to explain why there was not a single case at an infants' hospital where the milk was used. At the barns the milk was poured from the milking pails into large sterile cans and carried by wagon to the milk house where it was poured from the cans into the mixing tank. From the mixing tank it ran through the cooler and was bottled, the bottling going on continuously while fresh milk was being poured into the mixing tank. The apparatus was properly sterilized before each milking and the whole process carried on under very good conditions. There might be in the mixing tank the milk from not more than ten or fifteen cows at any one time. Barns B and C were within about 200 yards of the milk house and milk from these barns was therefore the first to enter the mixing tank. Milk from Barn A which was a quarter mile or more from the milk house was likely to enter the mixing tank toward the latter part of the milking. Hence, the larger part of the milk escaped contamination by the milk of Cow 108 which was in Barn A.

The Dairy Employees.

The theory advanced by Smith and Brown (1) that epidemic milk-borne tonsillitis is due not to the streptococci of ordinary bovine mastitis but to streptococci of human origin inoculated into the milk ducts of cows during milking has now received considerable support from the work of other authors.

In view of the recent remarks of Keegan (3) it seems necessary again to emphasize the fact that infection of the cow's udder by *Streptococcus epidemicus* may persist for some time without gross evidence of mastitis. That such an infection is not common is shown by the work of Jones (4) who studied over 80 cases of mastitis in cows without finding *Streptococcus epidemicus* except possibly in one instance. These facts make the finding of this organism in the udder or milk supply in association with an epidemic all the more significant. It was not the intention of Smith and Brown to deny the possibility of contact infection.

The theory of Smith and Brown offers a plausible explanation of the relative rarity of such milk-borne epidemics notwithstanding the wide prevalence of bovine mastitis, or garget, and its occurrence in

TABLE I.
Infected Individuals at the Dairy Farm.

Individual.	Employment and habitat.	Cultural history.	Clinical history.
W	Driver of automobile truck and miscellaneous work. Lived at farm boarding house.	<i>S. epidemicus</i> + Feb. 26, Mar. 20, Apr. 2, 9. Never present in large numbers. Negative cultures at intervals between above dates.	One tonsil large but no clinical symptoms at any time.
C	Milker. Lived at farm boarding house.	<i>S. epidemicus</i> + Feb. 26, 28, Mar. 1, 2, 5, 8, 10, 12, 13, 14. Often in considerable numbers. Negative cultures Mar. 15, 16, 17, 20, and thereafter.	Quinsy sore throat shortly before Feb. 26. No clinical symptoms after Feb. 26. Mar. 12 to 17. Daily irrigation of crypts of tonsils with hydrogen peroxide.
S	Milker and feed mixer. Lived at home. Father of K.	<i>S. epidemicus</i> + Mar. 2, 4, 5, 6, 8, 10, 12, 13, 14, 16. Often present in fair numbers. Negative cultures Mar. 17, 20, Apr. 2, and thereafter.	Large tonsils with large crypts but no clinical symptoms before Mar. 15. Mar. 12 to 17. Daily irrigation of crypts of tonsils with hydrogen peroxide. Mar. 15. Complained of rheumatism. Apr. 2. Confined to home for several days with rheumatism of back.
M	Milker. Lived at farm boarding house.	<i>S. epidemicus</i> + Mar. 2, 5, 6, Apr. 23, 30, May 14, 29, June 5, 11. At first present in considerable numbers; later very few. Negative cultures Feb. 26, 28, Mar. 3, 8, 10, 20, Apr. 2, May 7, 25.	No throat symptoms. Mar. 10. Complained of rheumatism. " 20. Confined to bed. Temperature. Left knee swollen and painful. Sent to hospital. Apr. 2. Back from hospital. Re-

<p>I</p> <p>Milker. Lived at farm boarding house.</p>	<p><i>S. epidemicus</i> + Mar. 8, May 14. Present in small numbers. Negative cultures Mar. 3, 5, 14, 20, Apr. 16.</p>	<p>ports involvement of both knees, left elbow, and both hands. Able to resume work with some discomfort. Apr. 27. Began use of autogenous vaccine of <i>S. epidemicus</i>. Gradual improvement.</p>
<p>K</p> <p>Milker. Lived at home. Son of S.</p>	<p><i>S. epidemicus</i> + Mar. 20, 23, 26. Present in fair numbers. Negative cultures repeatedly before and after above dates.</p>	<p>Left tonsil enlarged. Mar. 15. Complained of rheumatism. " 20 to Apr. 16. Unable to work at times. Puffy swelling of wrist and ankle. Apr. 24. Part of left tonsil cut off by doctor.</p>
<p>J</p> <p>Milker. Lived at home.</p>	<p><i>S. epidemicus</i> + Mar. 23, Apr. 2. Present in small numbers. Negative cultures Mar. 26 and after Apr. 2. No diphtheria bacilli found.</p>	<p>Had been quarantined at home with "diphtheria" for several weeks previous to Mar. 23. No swabs examined during that time. No subsequent clinical symptoms.</p>
<p>Miss V.</p> <p>Father and mother employed on farm. Lived at home.</p>	<p><i>S. epidemicus</i> + Apr. 30. Present in moderate numbers.</p>	<p>Said to have had several attacks of quinsy sore throat during past few months. The swab was taken just previous to removal of tonsils on Apr. 30. No other swabs taken.</p>

practically all dairy herds of any size. With this theory in mind our next effort was to determine the source of the infection of Cow 108. Unfortunately two of the milkers who had attended this cow at the time the epidemic started had left the employ of the dairy and it was impossible to obtain material or clinical data from them. Under the system of milking in use it was impossible to connect the act of milking any given cow with any particular milker.

On February 26 swabs were taken from the throats of fifteen employees, mostly milkers, at the dairy farm. All these men reported themselves in good health. One milker, C, had recently recovered from quinsy sore throat, but this man was said not to have worked in Barn A where Cow 108 was kept. *Streptococcus epidemicus* was found on swabs from C and another man, W, who drove the milk truck and did other odd jobs about the farm but did no milking. Both C and W lived at the farm dormitory and boarding house. Cultures from the other men were negative on this date. Swabs were taken and cultured from as many of the men as possible every few days. In Table I is given a tabular summary of the study of infected employees listed in the order of the discovery of the infection. Special attention is called to J, a milker, living at home, who had just returned to work after being under treatment for diphtheria for several weeks. Two women were employed at the farm boarding house. Swabs from both of these were examined repeatedly with negative results. One, Mrs. H., reported that she had "lost her voice several weeks ago as a result of laryngitis," but appeared well at the time of this investigation. The other, Mrs. V., had no clinical history. Her husband also was employed on the farm but gave no clinical history, and no positive cultures were obtained from him. On April 30, however, it was learned that a young daughter of Mr. and Mrs. V. was to be taken to Boston to have her tonsils removed. It was learned that this child had suffered from several attacks of quinsy sore throat during the past few months. She did not live on the farm but a swab from her throat was obtained previous to operation. Moderate numbers of *Streptococcus epidemicus* were found. It was also learned from K on March 26 that he had a young sister who "came near having pneumonia lately" and another sister who had frequent "earache due to adenoids." Swabs from these people could not be obtained.

Altogether swabs were studied, in most cases at regular intervals of a few days, from twenty-six persons connected with the dairy. From eight of these *Streptococcus epidemicus* was isolated at one time or another. At least two harbored the organism when the first swabs were taken on February 26.

The incidence of rheumatism among the infected men is noteworthy. There was no complaint of rheumatism among the non-infected employees.

From the data it seems impossible to connect any of these individuals with the introduction of the infection into the dairy and the infection of Cow 108. There are too many possibilities. It may be that C, or J, some member of the family of S, or Miss V. was the source of infection, or it may have been one of the men who left the dairy before the investigation was started. On the other hand, it seems likely that some of these and perhaps other persons were infected by using the milk of Cow 108, since all the employees and their families used the milk of the dairy. It is also possible that there was a good deal of contact infection, and there seems no other way to account for the infection of K, D, and possibly S, M, and J after Cow 108 had been removed. It is also possible that J's disease was a pseudodiphtheria due to *Streptococcus epidemicus*.

After Cow 108 had been eliminated from the herd frequent examinations of the milk and of swabs from the men's throats were made. All infected men were kept from milking or handling the milk in any way.

Data Bearing on the Mastitis of Cow 108.

Cow 108 gave birth to a calf in November, 1916. She was apparently normal and gave a good quantity of milk from each quarter of the udder up to the time of this investigation on February 24, 1917. On this date, as related above, *Streptococcus epidemicus* was found in the mixed milk from the four quarters of the udder. A photograph of the blood agar plate made at this time is shown in Fig. 1. Samples of milk from individual quarters of the udder showed infection in only the left fore quarter. On February 26 the milk from this quarter was noticeably thick and yellow. *Streptococcus epidemicus* was present in pure culture. Samples of milk from individual quarters were

again studied on March 11. By this time the left fore quarter was manifestly shrunken, the milk from it diminishing in amount and being quite thick and yellow. The culture was as before. Milk from

TABLE II.

Infected Cow 108.

Feb. 24. Mixed sample of milk from all four quarters. Gross appearance normal. Large numbers of *Streptococcus epidemicus* per cc. of milk. Cow isolated.

Date.	Milk from left fore quarter.		Milk from left hind quarter.	
	Gross appearance of milk.	<i>S. epidemicus</i> per cc.	Gross appearance of milk.	<i>S. epidemicus</i> per cc.
1917				
Feb. 25	Thick; yellowish.	Large numbers.	Normal.	None.
Mar. 11	“ yellow.	“ “	“	30,000 ±
“ 15			“	15,000
“ 15-20	Left hind teat injured, probably by being stepped on.			
“ 20			Curdled; yellowish.	872,000
“ 25	Cow removed to animal hospital.			
“ 26	Serous; yellow. 75 cc.	15,000	Normal. 275 cc.	375,000
“ 28	Watery. Few cc.	39,300	“ 375 “	200,000
Apr. 1	Serous; flocculent. Few cc.	22,000	“	23,000
“ 8	Serous. Few cc.	175,000	“ 250 cc.	28,500
“ 15	“ with white stringy particles.	32,000	“	57,000
“ 23	Serous; stringy. 10 cc.	90,000	14 million leucocytes per cc. Slightly flocculent. 250 cc.	75,000
May 2	Serous.	665,000	16 million leucocytes per cc. Normal.	32,000
“ 9	“ stringy. 5 cc.	57,500	“ 250 cc.	29,500
“ 16	Serous; stringy. 8 cc.	2,085,000	Slightly stringy. 250 cc.	60,000
June 6	Serous; thick yellow masses. 5 cc.	250,000,000	Normal. 150 cc. 5 million leucocytes per cc.	50,000
“ 20	Serous; thick yellow masses. 5 cc.	270,000,000	Slightly stringy. 150 cc.	2,000

the other three quarters was quite normal in appearance and amount. In the plate of milk from the left hind quarter, however, were found 30,000 colonies of *Streptococcus epidemicus* per cc. of milk. On gross

examination this quarter of the udder was apparently normal. On March 15 were found about 15,000 of the streptococci per cc. of milk from the left hind quarter. Some time between this date and March 20 the teat of the left hind quarter was injured. When examined on March 20 by one of us the teat was swollen and blood was dripping from it occasionally. There was a cut leading outward from the meatus. The milk collected at this time was thick, yellow, curdled, and slightly tinted with blood. Culture revealed 872,000 of the infecting organism in apparently pure culture per cc. of milk. Soon after this the cow was removed from the farm to the Angell Memorial Animal Hospital² where it was accessible for study from the laboratory at Harvard Medical School. To avoid transference of the infection from infected to normal quarters by the hands of the milker, he was instructed always to milk the normal quarters first. The milk from the four quarters was studied for many weeks. During this time the general condition of the cow was normal and the right fore and hind quarters of the udder remained uninfected. The results of the examination of the milk from the left fore and hind quarters are shown in Table II.

The point of greatest interest in as far as the epidemic is concerned is that there was detected bacteriologically an infection with *Streptococcus epidemicus* of the left fore quarter of the udder of this cow before it had attracted the attention of the herdsman and milkers at the dairy, that it was detected in the left hind quarter a week or more before the milk showed any gross change, and that the milk of this quarter again returned to almost normal appearance though the infection persisted for many weeks.

At the end of June the authors left Boston. Further study of Cow 108 was carried on by Dr. E. E. Tyzzer and Dr. Marshal Fabian.

Study of Cultures.

Although *Streptococcus epidemicus* was isolated 80 times from patients, dairy employees, and Cow 108, all the strains were indistinguishable from one another. A general description therefore suffices for all.

² The cow was kept in this hospital through the courtesy of Dr. S. J. Mixer of Boston.

Appearance in Blood Agar.—In the blood agar plate composed of standard beef infusion agar plus 5 to 10 per cent of horse blood, the deep colonies after 18 to 24 hours incubation are biconvex, usually a little larger than those of typical *Streptococcus pyogenes*, and surrounded by a distinct clear colorless zone of hemolysis about 2 to 2.5 mm. in diameter unless the plate is crowded with colonies. There are no intact corpuscles next to the colony and no discoloration. The appearance is that of the beta type. The hemolyzed zone of the deep colony is essentially like that of *Streptococcus pyogenes*, but it may be a little slower in developing. The surface colonies on the blood agar plate or slant serve to distinguish this organism from *Streptococcus pyogenes*. They are large colonies, 1 to 4 or 5 mm. in diameter, watery when grown in a humid atmosphere but drying down rapidly to thin transparent films when exposed to a dry atmosphere; *e.g.*, that of the room. If the surface colonies are close together, as in a streak, they become confluent and amebiform. Hemolysis appears more slowly about the surface colonies than about the deep ones, and in fact may not be very noticeable after incubation over night. This is partly due to the rapid overgrowth of the surface colony, obscuring the zone of hemolysis beneath. Not infrequently the surface colonies at this stage have a greenish tint which, however, is not due to the methemoglobinization of underlying blood corpuscles, though it may possibly be due to the formation of methemoglobin from released hemoglobin which has diffused into the substance of the colony. It is easily distinguished from the alpha type of appearance produced by pneumococci and *viridans* streptococci. The surface colony itself is like that of *Streptococcus (Pneumococcus) mucosus*, the Type III pneumococcus of Cole, but the latter organism produces the alpha appearance in blood agar and is further distinguished by fermentation and immunological reactions.

Morphology.—If some of the growth from a fresh watery surface colony is examined microscopically there are found diplococci and short chains of streptococci with large capsules enveloping the entire group. These capsules are best seen by suspending the material in a droplet of bouillon and a suitable India ink, covering with a cover-slip, and examining the moist preparation under the microscope. They are also revealed by the Huntoon capsule stain. The cocci themselves

are round or slightly flattened, closely packed together within the chain. They are Gram-positive and when seen encapsulated closely resemble *Streptococcus (Pneumococcus) mucosus*.

Appearance in Bouillon.—In bouillon there is nothing to distinguish *Streptococcus epidemicus* from *Streptococcus pyogenes*, though the former is likely to produce more clouding and less sediment than the latter. Usually the bouillon is fairly well clouded and there is a moderate amount of finely flocculent sediment which is easily disintegrated and suspended by shaking. Microscopically there are found moderately long chains of streptococci. A small amount of capsular substance may or may not be present.

Fermentation Reactions.—The fermentation reactions of all the strains were determined after incubation for 1 week in large test-tubes by titration of the total acidity of cultures in fermented bouillon plus 5 per cent of sterile horse serum and 1 per cent of the test substance.

In Table III the titratable acidity of representative strains is expressed as per cent normal acid. No subtraction has been made for the reaction of the medium. The fermentation reactions are the same as those of *Streptococcus pyogenes*. All the strains fermented saccharose, lactose, and salicin, but not raffinose, inulin, or mannite. It is to be assumed that they ferment dextrose and maltose also.

Comparison with Bovine Strains.—For comparison with the other strains there are included in Table III two strains of bovine hemolytic streptococci, H-Cow 71 and H-Cow 72. They produce a higher titratable acidity in saccharose and lactose media than do the strains of *Streptococcus epidemicus*. These two strains came from the milk of apparently normal cows. In blood agar they produce the beta type of hemolysis, but the zones show minor differences from those of the other strains. The zones of hemolysis develop rather slowly, those of H-Cow 71 remaining small, and those of H-Cow 72 becoming broad but with a hazy outer portion. Neither strain produces capsules. The individual elements are large. These minor differences between such non-pathogenic bovine strains and pathogenic hemolytic streptococci of human origin may easily escape the attention of one without considerable experience in the study of streptococci, but they are convincing when recognized. In 1915 it was said that:

"The success likely to attend the tracing of such epidemics to their source will depend upon a minute, detailed study of individual strains of streptococci and the discovery of certain minor distinguishing characteristics as guides" (1). This is still true and something

TABLE III.
Fermentation Reactions.

Strains from.	Titratable acid (per cent normal).					
	Saccharose.	Lactose.	Salicin.	Raffinose.	Inulin.	Mannite.
Patients.						
H-3	3.9	3.15	2.65	0.9	1.05	1.05
H-10	3.55	3.1	3.0	0.95	1.1	1.1
H-11	3.4	2.9	2.7	0.4	0.3	0.3
H-14	3.6	3.3	2.55	0.6	0.65	0.3
H-16	3.6	3.1	2.5	0.6	0.7	0.6
H-17	3.6	3.0	2.85	0.6	0.7	0.85
H-18	4.1	3.05	2.55	0.3	0.55	0.2
H-20	4.05	2.9	2.7	0.7	0.5	0.75
H-21	3.15	2.9	2.65	0.6	0.85	0.45
H-23	4.05	2.95	2.7	0.35	0.55	0.5
H-40	3.7	3.3	2.4	1.0	1.15	1.1
Employees.						
H-W	3.8	3.15	3.25	0.9	1.2	0.9
H-C	3.6	3.2	2.6	0.95	1.05	1.1
H-S	3.65	3.0	3.2	0.85	0.6	0.9
H-K	3.7	3.25	3.3	0.8	0.65	0.85
H-M	3.55	3.7	3.55	0.8	0.6	0.85
H-D	3.75	4.1	3.3	0.9	0.6	Lost.
H-J	3.6	3.0	2.55	0.6	0.5	0.4
Dairy cows.						
H-Cow 108	3.6	3.2	3.2	1.0	1.0	0.8
H-Cow 71	4.9	5.0	3.3	0.55	0.55	0.65
H-Cow 72	4.95	4.85	3.25	0.55	0.7	0.5

The titratable acidity of the medium was 0.5 to 1 per cent normal. A titratable acidity of less than 1.5 is regarded as a negative fermentation reaction.

further has been done to facilitate the recognition of these minor distinguishing characteristics. Ayers and his associates have called attention to the differences in final hydrogen ion concentration which serve to distinguish streptococci from different sources and many pathogenic from non-pathogenic streptococci (5, 6). Avery and

Cullen (7) have reported the usefulness of the determination of hydrogen ion concentration in the differentiation of hemolytic streptococci of human and bovine origin. They found that in dextrose bouillon "the human type of *Streptococcus hemolyticus* reaches a final hydrogen ion concentration of pH 5.2 to 5.0, and the bovine type of pH 4.5 to 4.3." Among the strains of streptococci which they studied were the three strains from dairy cows listed in Table III. Their results for these strains were as follows:

Designation of strain.		pH	Diagnosis.
Brown and Orcutt.	Avery and Cullen.		
H-Cow 108	V 10	5.1	Human type.
H-Cow 71	V 8	4.5	Bovine "
H-Cow 72	V 9	4.5	" "

The fact that streptococci of bovine origin produce in carbohydrate media more acid than do pathogenic streptococci of human origin has been noted by various authors (Broadhurst (8), Stowell, Hilliard, and Schlesinger (9), and Smith and Brown (1)) employing the titration method, and has been utilized as a means of differentiating streptococci from these two sources. In our experience more than 90 per cent of bovine streptococci of the beta type produce in dextrose bouillon from 1 to 1.5 per cent more normal acid than do human streptococci of the beta type, and the method of titrating the total acidity against 0.05 N sodium hydroxide with phenolphthalein as an indicator is reliable in the hands of an individual worker using a medium of fairly constant composition for the comparative study of strains from both sources.

Blood-Salt Solution Test.—In another article (10) is described the behavior of strains from this epidemic in a suspension of blood in salt solution. It is sufficient here to call attention to the fact that also by means of this test Strains H-Cow 71 and H-Cow 72 fall into the bovine group while Strains H-Cow 108 and H-M fall into the human group.

Animal Experiments.—Soon after isolation 1 cc. of bouillon culture of a number of strains from representative sources was injected intravenously into rabbits, with results as indicated in Table IV.

The organism was not particularly virulent as judged by the mortality of rabbits injected. The most conspicuous lesions produced were those of the external ears and testes. The lesions of the ears appeared on the 4th to the 9th day and resembled erysipelas, sometimes affecting the whole pinna simultaneously, but more often

TABLE IV.
Inoculated Rabbits.

Rab- bit.	Sex.	Culture.	Maximum temperature.	Weight (variation).	Localizations.	Result.
			^{°F.}	^{gm.}		
A	M.	H-10	105.5 (4th day).*	1,220-1,610	Apparently well throughout.	
B	"	H-C	107.3 (2nd day).	1,310-1,070	Both ears, 9th day. Left testis, 10th day.	Recovered (40 days).
C	"	H-S	105.1 (3rd day).	1,270-980	Right ear, 9th day. Lame in left hind leg temporarily, 10th day.	Recovered (40 days).
D	F.	H-M	106.0 (2nd day).	2,090-1,770	Endocarditis.	Died (4th day).
E	M.	H-M	106.6 (2nd day).	2,180-1,700	Both ears, 5th day. Both testes.	Very sick. Chloro- formed (7th day).
F	"	H-Cow 108	106.2 (2nd day).	1,330-1,150	Both ears, 4th day. Left eye, 4th " " testis, 4th day. Right testis, 14th day. Right hind foot, 14th day.	Died (29th day).
G	"	H-Cow 108 (19 mos. later).	106.5 (3rd day).	1,325-1,140	Left ear, 5th day. Right fore foot, 8th day.	Recovered (17 days).

* The day on which the injection was made is counted as the 1st day.

starting in one area and migrating to the remaining parts. The ear became red, hot, considerably swollen, and heavy so that it drooped. In a day or two there appeared little droplets of blood-stained serum exuding at points on either surface of the ear. When cultured in blood agar plates these droplets were found to contain large numbers

of the streptococcus injected. There often appeared areas of purpura, blisters or bullæ, and occasionally a necrosis, dry gangrene, and sloughing off of a part of the ear. If the rabbit lived the inflammation gradually subsided after a week or more, the skin became dry, underwent desquamation, and finally the ear regained its normal appearance. A section of an ear from Rabbit E revealed the following changes.

The cartilage and narrow strip of dense fibrous tissue on either side of it are intact. The looser vascular connective tissue and subcutis are greatly distended and in large areas obliterated by a serofibrinous exudate containing much cell debris (necrotic connective tissue and degenerating cell nuclei) but very few leucocytes and not many endothelial cells. In the tissue on the dorsum of the ear the blood vessels contain plugs of fibrin and some are plugged with streptococci, but most of the streptococci are massed in the lymph spaces and tissue spaces surrounding blood vessels. A few streptococci are seen scattered in the tissue spaces just beneath the cutis. The tissue on the ventral surface of the ear is less vascular and here the streptococci are seen quite generally scattered about throughout the distended connective tissue and fibrinous exudate. In the section studied the epidermis was intact and there were no blisters or bullæ.

It is to be noted that in three out of five rabbits both ears showed similar lesions, whereas the injection was made into the vein of only one ear. In no case did the infection appear to spread from the site of injection.

The next most common lesion was orchitis which occurred in three of five male rabbits injected with freshly isolated cultures. The scrotum enclosing the testis became hot, purplish red in color, swollen, and tense, the testis remaining in the sac. In Rabbit B the process subsided after a few days and there was a desquamation of the skin of the scrotum. In Rabbit F the orchitis persisted until death and at autopsy both testes were found adherent to the scrotum. The left testis which had been longest diseased was simply a homogeneous yellow caseous mass. The right one was a sac of fluid yellow pus. Cultures from the right testis revealed *Streptococcus epidemicus* in pure culture. In Rabbit E the orchitis was not detected until the animal was chloroformed on the 7th day. Both testes were freely movable from scrotum to abdomen but had a mottled appearance more noticeable in the left testis. This testis was sectioned and showed the following changes.

Transverse section of the testis shows on one side a distinct area of necrosis involving the tubules and interstitial tissue, and extending at one point into the tunica vasculosa. In the center of this area the outlines of the tubules are distinct and the parenchymatous cells are distinguishable but structureless and without nuclei. At the periphery of the necrotic area the appearance is that of coagulation necrosis with numerous disintegrating nuclei. Polymorphonuclear cells are scattered throughout most of the tunica vasculosa. On the opposite side of the testis from the necrotic area is a mass of fibrin containing in its meshes large numbers of polymorphonuclear leucocytes and large mononuclear cells. This mass is apparently outside the tunica vasculosa but was probably inside the tunica albuginea which has been stripped away from most of the testis and is not present in the section though remnants of it are seen. On one portion of the epididymis a fibrinous exudate between the tunica vasculosa and tunica albuginea is plainly seen, while scattered through the connective tissue of the epididymis are many polymorphonuclear cells. The rete testis and the ducts of the epididymis are apparently normal as is also the connective tissue of the septula and that between the tubules of the testis. A few streptococci are seen within leucocytes.

Other lesions encountered and studied were an endocarditis in Rabbit D and a keratitis and conjunctivitis in one eye of Rabbit F. Rabbit D apparently died suddenly as a result of the fresh cardiac lesion. One of the cusps of the tricuspid valve was enormously thickened, forming a large tumor that could be seen through the heart wall. A stained section showed an edematous and necrotic valve filled with fibrin and bearing a large subendothelial hemorrhage at its free edge. Small clumps of streptococci were visible in the tissue. Rabbit F had an infection of the cornea of one eye which spread outward into the conjunctiva and inward into the anterior and posterior chambers of the eye, also into the choriocapillaris and retina. *Streptococcus epidemicus* was isolated repeatedly from the conjunctiva during life.

The lesions of the ears and testes appearing in such a large percentage of the rabbits injected with cultures from this epidemic seemed remarkable and more than mere coincidence. In 52 rabbits similarly injected with streptococci from other epidemics by Smith and Brown similar lesions of the ears were encountered four times and orchitis not at all. The tendency to produce these lesions in rabbits was apparently one of the "distinguishing characteristics" of strains isolated in this epidemic, and one which tended to identify the strain

from Cow 108 with those from throats. In contrast to the results reported by Rosenow (11) at various times in the elaboration of his theory of "selective localization," however, it is to be noted that erysipelas and orchitis were not reported in any of the human patients during the epidemic. The organism produced certain characteristic lesions in patients, and others equally characteristic in rabbits. It was interesting to find that the strain from Cow 108, 19 months after isolation, was still able to produce the ear lesion in Rabbit G, but orchitis was not produced.

Subsequent Study of the Dairy.

The contamination of the milk with the streptococcus from Cow 108 was something which could not be detected by current routine methods for the examination of milk. One of our objects, therefore, was to devise methods for safeguarding the production of raw milk against such accidents. With this end in view milk from the cows of Dairy H and swabs from the throats of the employees were studied for 2 or 3 months after the subsidence of the epidemic. There is nothing particularly difficult about culturing throat swabs from the employees in blood agar once a week. This, we believe, is the most important measure for the prevention of milk-borne streptococcus epidemics. Second in importance is the culturing of milk in blood agar. Obviously it is not practicable to culture the milk of individual cows each week. It was found, however, that if a mixed sample of the milk from a group of ten to fifteen cows was plated out in blood agar it was easy to detect strains of bacteria which formed the characteristic flora of milk from individual cows of the group. In the examination of milk from individual cows it was found that certain cows gave almost sterile milk while others gave milk containing large numbers of bacteria. These high counters, as we called them, seemed to harbor a characteristic flora in their milk ducts. These organisms were commonly streptococci of the alpha or gamma type in blood agar, sometimes micrococci, and more rarely bacilli or streptococci of the beta type. A small amount of the milk of Cow 108 added to a group sample was easily detected in the blood agar plate. Many times we were able to detect the withdrawal of a cow or the addition of a cow

in a certain group by a change in the group flora. If an organism appeared in a group sample which was at all suspicious, samples from the individual cows of that group were cultured. By the use of this procedure it would be easy to lower the bacterial count of a dairy milk by gradually eliminating certain cows which are high counters. In the purchase of a dairy cow there is no reason why this should not be taken into consideration.

In correlation with the regular examination of throat swabs from the milkers and group samples from the cows, the confining of a certain milker to a certain group of cows is highly desirable from a sanitary standpoint. In Dairy H this was considered impracticable but we believe that the ability to fix the responsibility for a certain grade of milk from a certain group of cows with a certain milker would have economic as well as sanitary advantages.

As a minimum requirement for dairies producing raw milk we would recommend the regular examination of throat swabs from the milkers and the use of blood agar rather than plain agar for making milk counts.

SUMMARY.

A streptococcus epidemic of moderate extent and severity was characterized by clinical symptoms different from the usual septic sore throat, though the organism found was culturally *Streptococcus epidemicus*.

The infection was traced to the milk from a single quarter of the udder of a cow in a dairy of 112 cows producing an otherwise excellent grade of raw milk.

A number of the milkers on the dairy farm were found infected. It was impossible to trace the infection of the cow's udder to any one of the milkers, though such an infection seems probable since the streptococcus isolated from the cow was in every respect like streptococci isolated from patients and milkers, and different from those usually found in normal cows or cows with garget.

Certain recommendations are made to safeguard producers of raw milk against the occurrence of such epidemics.

Addendum.

The authors are indebted to Dr. E. E. Tyzzer and Dr. Marshal Fabyan of the Department of Comparative Pathology of Harvard Medical School for the following notes regarding Cow 108 after she had passed from under our observation.

Cow 108 was kept under observation until Jan. 3, 1918, when, since lactation had ceased, she was slaughtered for beef. During this time the following bacteriological examinations of the milk were made.

Aug. 27, 1917. Milk from the left hind quarter showed the hemolytic streptococcus previously isolated.

Aug. 29. Milk from the right hind quarter, no hemolytic organism present in plates.

Aug. 30. Milk from the right fore quarter, hemolytic streptococcus not present.

Sept. 11. Right hind quarter negative.

Sept. 13. Right fore quarter negative.

Sept. 25. Both right hind and right fore quarters negative.

The infected quarter was the first to become dry and the secretion of milk had ceased in other quarters by the middle of November. The localized induration which had become apparent soon after the cow came under observation, persisted up to the time of slaughter. It was situated at some distance above the teat in the anterior portion of the left hind quarter of the udder and appeared as an ill defined mass of about the size of a hen's egg.

Autopsy.—The udder was first sliced in various directions by the inspector who made his examination before turning the material over for further study. The affected portion after incision showed remarkably little difference from the normal portion. The involved tissue, however, was slightly firmer and less flabby than the normal gland. The animal showed also an early tuberculosis involving the bronchial lymph nodes and a small portion of the lung.

Microscopic Examination.—Stained sections of various samples of the mamma showed definite inflammatory changes in the indurated part, which were absent in other portions. There appeared to be an increase in the interglandular connective tissue, although this is rather difficult to determine and may be an open question. The chief abnormality consisted of collections of lymphoid cells mingled with which were few plasma and large mononuclear cells. The larger foci were distributed in the walls of the large ducts but there were also similar foci including gland acini. The ducts showed eosin-staining secretion in which an occasional mononuclear cell was apparent, and this material differed in no way from the secretion found in the ducts of the normal quarters.

Cultures taken from the indurated portion of the gland were negative. The inoculation of rabbits with a suspension obtained by grinding this tissue in salt solution resulted negatively.

It is evident from these findings that the infection was present in the left hind quarter as late as Aug. 27, 1917. At the time of autopsy no evidence was obtained of the presence of a virulent streptococcus. The absence of polynuclear leucocytes in both the tissues and the secretion indicates that there was at this time no active process present.

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EXPLANATION OF PLATE 9.

FIG. 1. The first blood agar plate culture of milk from Cow 108 showing large numbers of colonies of *Streptococcus epidemicus*, after incubation over night. The plate was too thickly seeded for the colonies and zones to attain their full development.

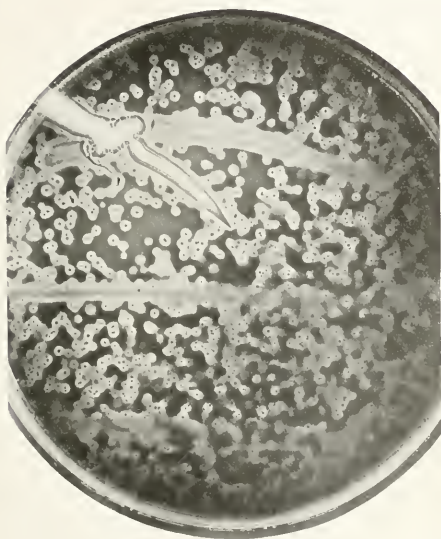


FIG. 1.

(Brown and Orcutt; Dairy infection with *S. epidermidis*.)

THE EFFECTS OF INTRAVENOUS INJECTIONS OF DI- CHLOROETHYLSULFIDE IN RABBITS, WITH SPECIAL REFERENCE TO ITS LEUCOTOXIC ACTION.*

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PLATE 10.

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INTRODUCTION.

There exists evidence pointing towards the general toxicity of dichloroethylsulfide both when administered by inhalation and when injected subcutaneously or intravenously.

Lynch¹ found that dogs gassed with high concentrations of dichloroethylsulfide (0.3 mg. per liter for 1 hour) exhibited symptoms similar to those produced by injection, and not referable to the primary irritation of the respiratory tract. These symptoms were salivation, vomiting, bloody diarrhea, hyperexcitability, and convulsions, with a slow irregular pulse which became rapid before death, and were attributed to vagal paralysis. Furthermore, the absorption of dichloroethylsulfide during inhalation was shown by the appearance of the hydrolysis product, dihydroxyethylsulfide, in the urine.

* The experiments were performed at the Experimental Field of the Chemical Warfare Service, American Expeditionary Forces, and were continued by one of us at the Brady Laboratory of Pathology of the Yale Medical School.

We are indebted to Lieutenant Colonel H. C. Clark, Chemical Warfare Service, for advice, to Professor M. C. Winternitz for the privilege of his laboratory, and to Professor F. P. Underhill for procuring samples of nitrobenzene and chlorobenzene.

A preliminary note of this work was presented at the meeting of the Society of Experimental Biology and Medicine, New York, March 19, 1919.

¹ Lynch, V., American University Experiment Station, Monograph No. 1, Washington, 2nd edition, 1918, 317. See also Lynch, V., Smith, H. W., and Marshall, E. K., Jr., Dichlorethylsulphide (mustard gas). I. Systemic effects and mechanism of action, *J. Pharmacol. and Exp. Therap.*, 1918-19, xii, 265.

Severe symptoms, ending usually in death within 24 hours, followed the intravenous injection of approximately 0.01 gm. per kilo in dogs. At autopsy intense congestion, often with extensive hemorrhage into the intestinal mucosa, was the only lesion noted.²

Muratet and Fauré-Fremiet³ reported upon blood examinations in a series of six rabbits, four of which were poisoned by inhalation and two by subcutaneous injection. In all the animals the following changes were noted. The red blood cells were augmented in number soon after exposure (increase to 6,000,000 to 10,000,000), and then gradually diminished. Nucleated forms were sometimes seen. There was no polychromatophilia or other degenerative change. The leucocytes showed a rise following the intoxicating dose, but later diminished progressively. There was a relative lymphocytosis. Morphologically, degenerative changes were found in the leucocytes. The polymorphonuclears showed abnormal lobulation of the nucleus, dissolution of the granules, and, finally, a breaking up of the chromatin into spherical masses. The lymphocytes also showed degenerative changes, and many abnormal cells which could not be identified were present in the films. Jolly⁴ repeated the experiments of Muratet and Fauré-Fremiet, using both dogs and rabbits, and administering the dichloroethylsulfide both by inhalation and subcutaneous injection. His results were less consistent than those of the previous workers, but a study of the figures obtained seems to show a rather constant diminution of leucocytes after injection; with inhalation the results were variable.

Zunz⁵ observed in severely gassed human cases a leucopenia with relative lymphocytosis developing after several days.

Stewart,⁶ in a study of the blood changes in six fatal cases of poisoning with mustard gas, found an initial polymorphonuclear leucocytosis, followed by a rapid fall in the total and neutrophil count, beginning on the 3rd or 4th day, and reaching an extremely low figure (200 to 500) before death. The leucopenia is ascribed to acute failure of the leucoblastic function, without associated erythroblastic failure. No histological study of the bone marrow is included in the report.

² Winternitz, M. C., Finney, W. P., and Wislocki, G. B., American University Experiment Station, Monograph No. 1, Washington, 2nd edition, 1918, 389.

³ Muratet, L., and Fauré-Fremiet, Confidential Report of the French Gas Service.

⁴ Jolly, Confidential Report of the French Gas Service.

⁵ Zunz, Report to the Interallied Gas Conference, Paris, 1918.

⁶ Stewart, M. J., Report on cases of poisoning by "mustard gas" (dichloroethylsulfide) with special reference to the histological changes and to alterations in the leucocyte count, Report of the Chemical Warfare Committee, *Great Britain Med. Research Com. Rep., No. 17*, 1918.

Krumbhaar⁷ also reported a series of human mustard gas cases in whom there developed, after the initial leucocytosis, a marked leucopenia, persisting even in the presence of the complicating bronchopneumonia. Moderate anemia, without blast formation, and an apparent decrease in the number of platelets were also observed. Postmortem examination of the bone marrow showed a "greater or less disappearance of normoblasts, myelocytes and adult forms," only "primordial cells and megaloblasts" remaining. Krumbhaar's observations offer convincing evidence of a systemic effect in severe cases of mustard gas inhalation. It is highly improbable that the leucopenia and other blood changes were due to complicating influenzal infections, inasmuch as practically all these gas cases occurred previous to the development of the influenza epidemic.

Hermann,⁸ in his study of the clinical pathology of mustard gas poisoning, finds "no leucopenia at any time," the changes observed being slight secondary anemia with a well marked polymorphonuclear leucocytosis, a definite eosinophilia, and the appearance of myelocytes and young forms of leucocytes. The value of Hermann's observations is lessened by the fact that in none of the eight cases upon which his report of the blood changes is based, was the blood examined before the 10th day after gassing, and that only one of his cases was fatal, and the majority mild in character.

Warthin and Weller⁹ studied the effects of subcutaneous and intravenous injection of dichloroethylsulphide in a series of rabbits and dogs. The toxic symptoms described are salivation, diarrhea, depression of temperature, and increased rapidity of respiration followed by slowing. With large doses animals, after a short period of nervous excitement, die within a few hours in coma with gradual failure of respiration. The gross lesions, apart from the local effect, consist of a general passive congestion of all organs with minute hemorrhages, emboli, and infarctions. The only specific changes are more or less severe catarrhal enteritis with corresponding microscopic findings. A striking microscopic feature is the presence of hemorrhages and pigmented phagocytes in great numbers in the splenic tissue. Intravenous injections (dose of 0.0075 to 0.18 cc.) are followed, after a quiescent period, by general convulsions, opisthotonos, irregular jerking movements, salivation and lacrimation, and fall of temperature. The animal passes into coma, and there is gradual respiratory and cardiac failure. Patho-

⁷ Krumbhaar, E. B., Rôle of the blood and the bone marrow in certain forms of gas poisoning. I. Peripheral blood changes and their significance, *J. Am. Med. Assn.*, 1919, lxxii, 39.

⁸ Hermann, G. R., The clinical pathology of mustard gas (dichlorethylsulphide) poisoning, *J. Lab. and Clin. Med.*, 1918-19, iv, 1.

⁹ Warthin, A. S., and Weller, C. V., The general pathology of mustard gas (dichlorethylsulphide) poisoning, *J. Lab. and Clin. Med.*, 1919, iv, 265; also in *Researches on the pathology of mustard gas (dichlorethylsulphide) poisoning*, Contributions from the Pathological Laboratory, University of Michigan, 1918-19, ix.

logically, there are found dilatation of the right side of the heart, congestion, petechia, hemorrhages of the lungs, and general congestion of other viscera. We have found no reference in Warthin and Weller's papers as to changes in the circulating leucocytes or bone marrow except the general statement¹⁰ that animals exposed to direct application in the gassing chamber may show, among other effects, "secondary anemia, leucocytosis or leucopenia."

The following report deals with the effects of the intravenous injection of dichloroethylsulfide in rabbits. We have given special attention to the alterations in the blood picture and in the blood-forming organs, since these were the most striking of the results observed.

Technique.

Method of Injection.—Difficulty was at first experienced in obtaining suitable emulsions for injection. In the earlier experiments the given amount of a 10 per cent alcoholic solution by weight of dichloroethylsulfide was suspended in 0.85 per cent salt solution, shaken vigorously, and immediately injected, after allowing the larger globules to settle out. The dose administered was, therefore, considerably less than the total amount taken. Later it was found that a more satisfactory suspension could be made by emulsifying in 30 per cent alcohol in distilled water. A slightly milky emulsion was obtained which after shaking did not separate out in the time necessary for injection. The suspension was prepared from a recently made, accurately weighed 10 per cent solution in absolute alcohol, and immediately injected to avoid hydrolysis. The dichloroethylsulfide used was a distillate from the contents of a German yellow cross shell and was actively vesicant.

Dose.—We have not attempted to determine accurately the minimum lethal dose. It was found in the earlier experiments that the limit of tolerance was in the neighborhood of 0.01 gm. per kilo, and in the last six rabbits used a uniform dose of 0.005 gm. per kilo was chosen.

¹⁰ Warthin, A. S., and Weller, C. V., The general pathology of mustard gas (dichlorethylsulphide) poisoning, *J. Lab. and Clin. Med.*, 1919, iv, 266.

Symptoms.

Emaciation and Loss of Weight.—These symptoms were noted in all the rabbits which survived for 2 days or more after the injection. In all but one animal (Rabbit 1, loss of weight only 100 gm.) this was associated with diarrhea, and perhaps was due to it.

Nervous Symptoms.—These were observed only in Rabbits 2 to 4, which died 1 hour, $1\frac{1}{2}$ hours, and during the night following injection. The animals showed extreme restlessness, incoordinate movements, retraction of the head, and transient spasticity, but no definite paralyses or convulsions.

Respiratory Symptoms.—No definite or characteristic respiratory symptoms were observed even in animals dying a few hours after the injection, in which the occurrence of pulmonary embolism might have been suspected.

Intestinal Disturbances.—Diarrhea occurred in six animals, in all but one associated with the finding of gross lesions of the intestinal tract at autopsy. The feces were copious, fluid, dark brown, and not grossly admixed with blood or mucus.

Edema.—Edema of the ears of wide extent invariably followed injection, even when great care was taken to avoid introducing the material outside the vein. It would appear that the dichloroethylsulfide diffused readily through the vessel wall. It was also observed that after the injection was begun an area of blanching, involving the neighboring skin over a width of several centimeters, at once appeared. This persisted for a few minutes after the injection, after which the normal circulation was restored.

Pathology.

Respiratory Tract.—Of twelve rabbits injected, four showed definite pulmonary lesions (Nos. 2 to 5). All these animals died or were killed within a period of from 1 to 22 hours following the injection, and none of the eight animals surviving over 24 hours showed significant gross or microscopic lesions.

The changes observed were (1) irregular areas of edema, in part fibrinous; (2) areas of atelectasis and emphysema; and (3) accumula-

tions of leucocytes in the capillaries, often showing caryorrhexis and fragmentation, and slight emigration into the alveoli. The trachea and bronchi were normal except that they contained a homogeneous coagulum. No thrombi were found in the capillaries or larger vessels. The small pulmonary arteries were thick walled and appeared contracted. Clear vacuoles were seen beneath the endothelium. The significance of this finding is not clear as somewhat similar pictures may be seen in normal animals.

It is, of course, not possible to conclude from the histological findings that dichloroethylsulfide is eliminated by the pulmonary epithelium; on the other hand, no support is found for the view that the lesions are the result of capillary embolism due to impaction of dichloroethylsulfide globules. The fact that animals which survived for a longer period showed no pulmonary lesions might suggest that the edema is the result of a direct and immediate action of the dichloroethylsulfide upon the pulmonary capillaries.

Alimentary Tract.—No lesions were found in the esophagus. Rabbit 5 showed hemorrhages into the pyloric portion of the stomach and in the duodenum. Of the remaining rabbits, three (Nos. 6, 7, and 8) had a severe diphtheritic enteritis affecting the middle or lower portion of the intestine. Rabbit 6 showed also patches of membranous inflammation in the large intestine. Rabbit 9 also had a diphtheritic colitis, but this proved to be coccidial in origin. Seven rabbits were free from lesions of the intestinal tract.

Liver.—The liver showed no significant changes.

Nervous System.—No detailed study has been made. Neither capillary thrombosis nor hemorrhage was found.

Kidneys.—Definite changes were present only in the kidneys of Rabbits 2 and 5. The capsular spaces contained hyaline globules and occasionally red blood cells. Hyaline material was also present about the blood vessels in the intermediate zone between cortex and pyramids. Blood cells and hemoglobin (?) casts were found in the collecting tubules. The urine was not examined.

Blood and Hematopoietic System. General Considerations.—Since the observations of previous workers upon the blood changes following the administration of dichloroethylsulfide had yielded somewhat conflicting results, it was decided to undertake a detailed study of a

small series of rabbits, eliminating as far as possible incidental factors which might influence the blood picture. Of these incidental factors, variations in the surrounding temperature were found to be the most disturbing. When rabbits were exposed to a temperature of from 40–50°C. for $\frac{1}{2}$ hour, the total leucocytic count showed a tendency to fall, although there were individual exceptions. Counts made shortly after removing the animals from the warm chamber to room temperature (15–20°C.) invariably showed an abrupt and striking rise (Table I). After this point had been established, the rabbits were kept in the laboratory during the observation period and following the injection in order to avoid sudden chilling or abrupt temperature change. Daily counts were made at approximately the same time (9 to 11 a.m.) before food was given. The differential counts are based upon an enumeration of 500 cells, except when the extreme leucopenia made this impracticable. In spite of the usual precautions unexplained variations occurred which made it difficult to draw conclusions as to the percentile fluctuation. In making the counts blood was always taken from the ear not used for injection. When both ears had been used, a small cut was made in the skin of the abdomen and blood taken from one of the superficial abdominal veins. At least two counts were made on each rabbit before injection.

Erythrocytes.—Stress of other work prevented a detailed study of the numerical variation in the erythrocytes. Preliminary observation (Rabbit 9) showed no significant change. There were no striking morphological changes pointing to a marked anemia, except, perhaps, the occurrence of a moderate anisocytosis in the terminal stages. Polychromatophilia is not infrequently seen in films from normal rabbits. Rabbit 13, during the period in which recovery from the effects of an injection was taking place, showed numerous normoblasts—an indication that the erythroblastic tissue had suffered injury, as well as the leucoblastic. Histological study of the bone marrow and spleen affords further evidence of the toxic action of dichloroethylsulfide upon the formation of red blood cells, which will be described.

Leucocytes.—In all but two rabbits (Nos. 8 and 12) of those surviving more than 24 hours, there occurred after a single injection of 0.005

to 0.01 gm. of dichloroethylsulfide per kilo a pronounced fall in the number of circulating leucocytes. In these two rabbits a second injection of the same dose after 7 and 8 days respectively was followed by the typical reaction. The leucopenia was preceded by a transient rise in only one rabbit (No. 7), but it should be noted that no counts were made at short periods following the injection. Although degenerated leucocytes with poorly staining and fragmented nuclei and vacuolated cytoplasm were occasionally found in films, they were infrequent, and even in the presence of an extreme leucopenia the rare leucocytes present in the films were usually normal morphologically. Blood platelets were found at all stages and showed no alterations. As regards the behavior of the different types of leucocytes, a study of these data shows that in some instances the injection is followed by an absolute and percentile increase in the polymorphonuclears, which fall rapidly with the onset of the leucopenia. In the terminal stages the polymorphonuclears practically disappear from the peripheral blood. On the other hand, sometimes an initial fall in the number and percentage of polymorphonuclears is followed by an absolute and relative increase. This secondary rise is associated with the appearance of unripe forms in considerable numbers (Rabbits 12 and 13), and coincides with regenerative activity of the bone marrow, as shown by a study of sections from rabbits killed at this stage. The leucopenia is accompanied by a relative lymphocytosis. The absolute number of lymphocytes is diminished in the later stages and lags behind that of the granular cells in the cases in which regeneration is occurring. There is a percentile increase in the large mononuclear cells, but their absolute number is unchanged or diminished.

The data showing the alterations in the blood count are presented in Table I and in Text-figs. 1 to 3.

Bone Marrow.—Although the appearance of the marrow varies in different animals, there is evidence of the destructive effect of the dichloroethylsulfide upon the blood-forming elements. The variations observed can be correlated with different stages of injury and repair, and these again are reflected more or less closely in the blood picture during life. The following brief descriptions will illustrate the different phases observed.

Rabbit 1 died 4 days after injection. At the time of death the leucocyte count had fallen to 800, of which 46 per cent were polymorphonuclears. The marrow of the femur contains a large amount of adipose tissue, the fat cells being separated by a loose edematous tissue containing less than the normal number of cells. The leucocytes of the granulocyte series are almost without exception degenerated. The cytoplasm in sections stained with Wright's stain (normal control) shows no granules. The nuclei stain diffusely and are frequently fragmented. The megacaryocytes also show degenerative changes. Some contain clumps of pink-staining, hyaline material in their cytoplasm. There are islands of apparently normal erythroblasts. The blood sinuses are wide and intensely congested with normal appearing red blood cells. They contain practically no nucleated elements. The appearances are interpreted as indicating an active injury to the bone marrow. The toxic action seems to have affected especially the granular cells.

Rabbit 8 died 4 days after a second injection of dichloroethylsulfide. Leucocytes on the last 2 days had fallen to 800, a film of the peripheral blood showing very few nucleated cells, which were almost exclusively large and small mononuclears. Sections of the marrow show an extreme aplasia, comparable with that seen in experimental benzene poisoning. Myelocytes, polymorphonuclears, and megacaryocytes have practically disappeared. There are loose collections of normoblasts scattered through the edematous fat tissue. Occasional globular fragments of chromatin, often enclosed in phagocytes, represent the remains of the destroyed cells. The sinuses are congested and contain no leucocytes (Fig. 1).

Rabbit 12 was killed on the 7th day following a second injection. The leucocytes which had fallen to 1,000 on the 3rd day after injection then rose to 1,400 and 2,100, and at the same time large numbers of myelocytes appeared in the peripheral blood. The histological picture shows the effect of a previous injury and at the same time an active regeneration. The predominant cell type is the myelocyte, the granules of which are definite and well stained in Wright's preparations. There are also a fair number of adult polymorphonuclears. The myelocytes are congregated in islands, as are also the erythroblasts. Megacaryocytes are numerous and are not

3.00 p.m. Sept. 17, 9.00 a.m. " 18 " 19 " 20, 10.30 a.m. 3.00 p.m.				5,680,000	70	4,600 4,900 6,400 1,800 900 400	24 Practically all mononuclears.	430 58	1,050 18	320	
4	Sept. 9 " 10 " 12, 9.00 a.m. 10.35 " 11.30 " " 13, 9.00 " 10.30 " 11.00 " " 14, 9.00 " 10.30 " 11.30 " " 15, 9.00 " 10.30 " 11.30 " " 16, 2.00 p.m. 2.30 " 3.00 p.m. 10.00 "	13 40 18 11 30-40 13 13 50 16 17 Warm room (?). 19	0.01			17,400 15,300 17,500 8,800 24,200 8,000 13,600 20,000 11,700 6,800 26,200 12,400 19,200 10,400 12,300 15,400 8,200	56 11 54 41 72 43 68 52 50 41 22 50 25 42 Died.	9,750 39 1,680 70 9,450 40 53 22 3,440 50 4,000 7 7 6	6,780 5 10,710 19 7,000 6 4,000 7 7 6	870 2,910 1,050 560 6	
6	Sept. 12, 9.00 a.m. 10.35 " 11.30 "	13 40-56 18				12,000 5,800 18,200	58 70 61	6,960 40 27 37	4,800 2 3 2	240	

Where the hour is not given the blood counts were made at 9 to 9.30 a.m.

[illegible]

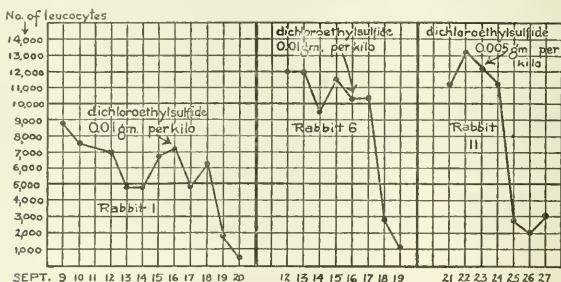
TABLE I—Concluded.

Animal No.	Date.	Temperature.	Dose per kilo.	Red blood cells.	Hemoglobin.	Total No. of leucocytes.	Polymorpho-nuclears.		Lymphocytes.		Large mono-nuclears.		Other forms.
							Per cent.	No. per c.mm.	Per cent.	No. per c.mm.	Per cent.	No. per c.mm.	
12	1918 Oct. 4 " 5 " 7	°C.	gm.		per cent	1,000 1,400 2,100	1 2 50	10 28 1,050	82 68 28	820 967 586	17 3 6	170 42 216	Myelocytes 27 per cent. Myelocytes 16 per cent.
8	Sept. 28 " 29 " 30 Oct. 1, 9.00 a.m. 11.00 " 3.30 p.m. Oct. 2 " 3 " 4 " 5 " 6 " 7 2.00 p.m.		0.005			9,800 10,200 8,000 5,000 11,000 11,800 9,600 10,000 14,500 13,800 13,200	20 48 22 52 70 56 61 64	1,960 4,900 1,760 5,000 7,000 8,100 8,420 8,450	76 44 64 40 25 40 30 34	7,450 4,490 5,120 3,840 2,500 5,800 4,140 4,490	4 8 14 8 5 4 9 2	390 810 1,120 768 500 580 1,240 260	
	Oct. 8 " 9 " 10 " 11		0.005			9,000 900 800	94 Died.	8,460	5	450	1	90	Large mononuclears too few to count.

[illegible]

degenerated. There are many mitoses. The marrow on the whole is less cellular than normal marrow, and new fat cells are in process of formation. There is much hemosiderin pigment, chiefly intracellular, which may be taken as evidence of previous blood destruction.

After the first injection in Rabbit 13 there ensued a fall in the leucocytes from 37,000 to 2,700 on the 4th day. This was apparently followed by an active regeneration. The count rose again to 32,400, followed by a slight drop to 25,400. At this point a second injection was given, which again was followed by a marked leucopenia, the leucocytes falling to 300 per c.mm., at which point the rabbit was

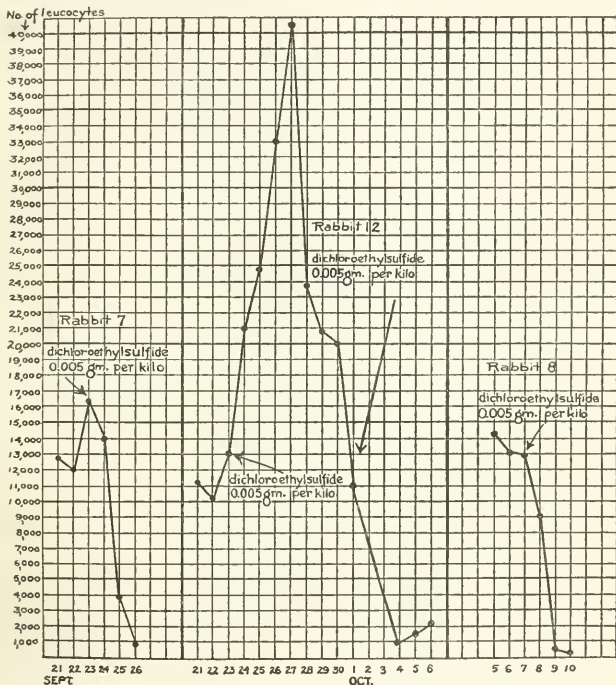


TEXT-FIG. 1. Variations in the total leucocyte count after the intravenous injection of dichloroethylsulfide.¹¹

killed. The marrow histologically shows evidence of an initial injury, followed by repair and a fresh destruction of the regenerating cells. There are large areas of almost complete aplasia, similar to that described in Rabbit 8. But there are also hyperplastic areas, composed of aggregates of large cells with poorly staining nuclei, which, under the high power, are found to be distorted and obviously degenerating. It would seem that these hyperplastic foci have been again injured by the second injection of the toxic substance.

¹¹ The text-figures include only the animals in which blood counts were made during a preliminary period. The fluctuations produced by abrupt change in the surrounding temperature are omitted.

Spleen.—Changes in the follicles—fragmentation of lymphoid cells with phagocytosis of chromatin particles—were seen only in Rabbits 4 and 5. Both these animals died within less than 24 hours after

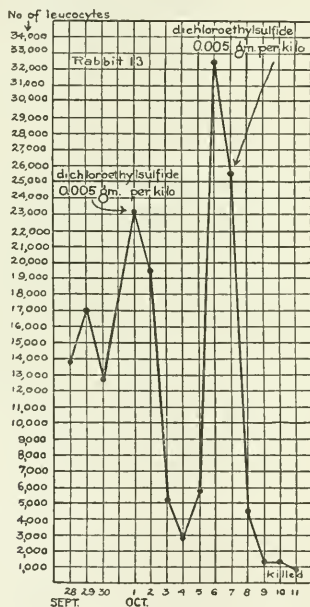


TEXT-FIG. 2. Variations in the total leucocyte count after the intravenous injection of dichloroethylsulfide.

injection. In the remaining rabbits the follicles were normal, or at least showed no signs of either active destruction or excessive proliferation. The sinuses in the majority of the rabbits contained large mononuclear cells laden with blood pigment. The most striking

change, however, was the paucity of free cells in the meshes of the reticulum. The sinuses were separated by strands of cells with pale oval nuclei, evidently belonging to the reticular elements.

Lymphoid Tissue.—The two rabbits (Nos. 4 and 5) which showed acute destruction of the lymphoid cells of the splenic follicles also



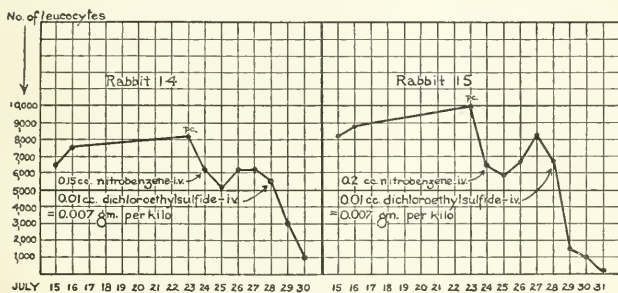
TEXT-FIG. 3. Variations in the total leucocyte count after the intravenous injection of dichloroethylsulfide.

showed cytolysis of the lymphocytes of the thymus and of the intestinal lymphoid tissue.

Because of the great susceptibility of the small thymus cells and of the tissue lymphocytes generally to injurious agents, we are not inclined to regard this as a specific effect of the dichloroethylsulfide,

particularly as it was not present when the destruction of the bone marrow elements was extreme.

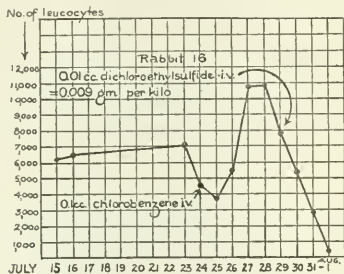
The injury to the blood-forming tissues described bears a close resemblance to the well known action of benzene, with the possible difference that the erythroblastic tissues appear to be less seriously injured than they are with the latter substance. It had been ascertained that the Germans were using nitrobenzene and chlorobenzene as solvents for dichloroethylsulfide in shell fillings, and, indeed, the distillate used by us in our experiments was said to have contained a proportion estimated at roughly from 10 to 20 per cent of these benzene derivatives. Although we were not able to find state-



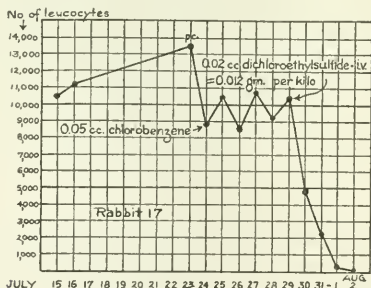
TEXT-FIG. 4. Variations in the total leucocyte count after the intravenous injection of nitrobenzene and dichloroethylsulfide.

ments in the literature as to the leucotoxic action of chlorobenzene and nitrobenzene, doubt naturally arose as to whether the effects which we observed might not have been attributable to the admixture of these solvents, rather than to the dichloroethylsulfide itself, although the minute amounts contained in the doses injected made this rather improbable. The armistice prevented further control experiments at that time. Recently, however, one of us has been able to perform additional experiments on a small series of rabbits, which make it clear that the toxic action upon the leucocytes and bone marrow is due to the dichloroethylsulfide, and not to a possible admixture of chlorobenzene and nitrobenzene.

After a preliminary period of observation two rabbits were injected intravenously with 0.15 and 0.2 cc. of nitrobenzene respectively—doses approximately fifteen and twenty times as large as the total dose of dichloroethylsulfide used in the original experiments. In



TEXT-FIG. 5. Variations in the total leucocyte count following the intravenous injection of chlorobenzene and dichloroethylsulfide.



TEXT-FIG. 6. Variations in the total leucocyte count following the intravenous injection of chlorobenzene and dichloroethylsulfide.

neither of the rabbits did a leucopenia develop. After 4 days, during which time the animals gained in weight and showed no symptoms, they received an intravenous injection of dichloroethylsulfide.¹² In

¹² The sample used was obtained by Dr. Winternitz from the American University, and was stated to be free from nitrobenzene and chlorobenzene.

TABLE II.

Animal No.	Date.	Substance injected.	No. of leucocytes.	Weight.
	1919			gm.
16	July 15, 1.00 p.m.	0.1 cc. of chlorobenzene intravenously.	6,175	1,600
	" 16, 2.10 "		6,600	
	" 23, 1.25 "		7,500	
	" 24, 11.10 a.m.		4,750	
	11.15 "			
	" 25, 10.30 "		3,680	1,650
	" 26, 10.00 "		5,500	1,530
	" 27, 10.00 "		10,250	1,670
	" 28, 10.15 "		10,400	1,540
	" 29, 10.00 "		7,900	1,500
	10.50 "	0.01 cc. of dichloroethylsulfide intravenously (0.009 gm. per kilo).		
	" 30, 10.30 "		4,800	1,470
	" 31		2,850	1,450
	Aug. 1		450	1,430
14	" 2			Found dead.
	July 15, 11.30 a.m.	0.15 cc. of nitrobenzene intravenously (1.5 cc. of 10 per cent solution in absolute alcohol plus 3 cc. of distilled water).	6,500	
	" 16, 1.30 p.m.		7,400	
	" 23, 1.15 "		8,100	
	" 24, 9.50 a.m.		6,250	
	10.05 "			
	" 25, 10.00 "		5,300	1,630
	" 26, 9.30 "		6,200	1,730
	" 27, 9.30 "		6,300	1,790
	" 28, 9.30 "		5,650	1,620
	2.35 p.m.	0.01 cc. of dichloroethylsulfide intravenously (0.007 gm. per kilo).		
	" 29, 9.30 a.m.		3,500	1,620
	" 30, 9.30 "		800	1,550
	2.30 p.m.			Killed.

TABLE II—*Concluded.*

Animal No.	Date.	Substance injected.	No. of leucocytes.	Weight.
	1919			gm.
15	July 15, 12.30 p.m.	0.2 cc. of nitrobenzene intravenously.	8,300	
	" 16, 1.45 "		8,850	
	" 23, 1.00 "		10,300	
	" 24, 10.30 a.m.		6,400	1,720
	10.35 "			
	" 25, 10.15 "	0.01 cc. of dichloroethylsulfide intravenously (0.007 gm. per kilo).	5,800	1,770
	" 26, 9.45 "		6,700	1,900
	" 27, 9.45 "		7,800	1,860
	" 28, 9.55 "		6,100	1,770
	" 29, 9.45 "		1,500	1,720
	" 30, 9.45 "		1,050	1,720
	" 31, 11.00 "		>200	?
			(counted from heart's blood).	Killed.
17	" 15, 12.45 p.m.	0.05 cc. of chlorobenzene intravenously.	10,450	
	" 16, 2.45 "		11,300	
	" 23		13,900	
	" 24, 11.30 a.m.		8,900	2,000
	11.35 "			
	" 25, 10.45 "	0.02 cc. of dichloroethylsulfide intravenously (0.012 gm. per kilo).	10,400	2,120
	" 26, 10.10 "		8,000	2,270
	" 27, 10.20 "		10,700	2,430
	" 28, 10.30 "		8,700	2,140
	" 29, 10.30 "		10,400	2,040
	11.00 "			
	" 30, 10.45 "		4,900	1,980
	" 31, 10.00 "		2,400	1,940
	Aug. 1, 10.15 "		300	1,900
	" 2, 10.00 "		>200	1,760
				Killed.

each rabbit the injection was followed by the typical leucopenia (Text-fig. 4, Table II).

Two rabbits also were injected with chlorobenzene, the doses used being 0.1 and 0.05 cc. respectively. One of these rabbits before injection showed a slight leucopenia (4,750 per c.mm.), and on the following day the leucocytes fell to 3,680; they then rose to slightly above normal and remained so. The other rabbit showed no significant fluctuation. 5 days after the first injection each rabbit received an intravenous injection of dichloroethylsulfide, the dose being 0.009 and 0.012 gm. respectively, and this was followed by the typical reaction. The leucocyte counts are shown in Text-figs. 5 and 6 and Table II.

These experiments, though not numerous, are clear-cut, and seem to rule out definitely the possible part played by the benzene derivatives in producing these striking effects upon the blood-forming organs.

CONCLUSIONS.

1. The lethal dose of dichloroethylsulfide (distilled from a German yellow cross shell), when injected intravenously into rabbits, is from 0.005 to 0.01 gm. per kilo.

2. Rabbits dying within 24 hours showed extensive hemorrhages and edema of the lungs.

3. Severe lesions of the intestinal tract were present in about one-third of the rabbits.

4. Dichloroethylsulfide injected intravenously is specifically poisonous for the hematopoietic tissues. Severe lesions are caused in the bone marrow, and the number of circulating leucocytes is markedly diminished. In animals surviving the injection regeneration occurs. The granular cells of the bone marrow seem to be more sensitive than the lymphoid cells and the erythrocytes.

5. The effect upon the blood and hematopoietic tissues is not due to the admixture of nitrobenzene or chlorobenzene in the shell filling. Injection of these substances in animals in amounts many times greater than the total dose of dichloroethylsulfide used produced no changes in the blood picture, and the subsequent injection of dichloroethylsulfide free from these solvents produced a typical reaction.

EXPLANATION OF PLATE 10.

FIG. 1. Rabbit 8. Bone marrow of femur 4 days after the second injection of 0.005 gm. of dichloroethylsulfide. The sinuses (*bld. ves.*) are widely dilated and filled with erythrocytes. They contain practically no nucleated cells. The endothelial cells (*end.*) are preserved. There are scattered megacaryocytes (*mg.*) with darkly stained nuclei. There are also degenerating cells with fragmented chromatin (*pyc.*). Myeloblasts, myelocytes, and adult granulocytes of all types have disappeared, and there are only occasional small mononuclear elements. The space between the sinuses is occupied by adult and immature fat cells (*f.*).

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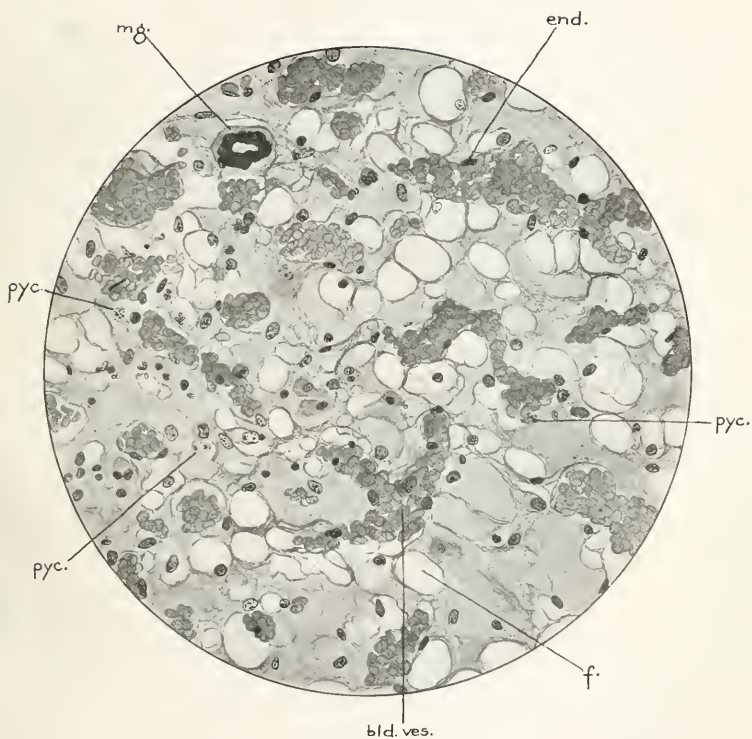


FIG. 1.

(Pappenheimer and Vance: Intravenous injections of dichloroethylsulfide.)

FUNGOUS DEVELOPMENTAL GROWTH FORMS OF BACILLUS INFLUENZÆ.

A PRELIMINARY NOTE.

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PLATES 11 AND 12.

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The problem of the causative agent of epidemic influenza, and especially that of the importance of Pfeiffer's *Bacillus influenzae*, until recently generally believed to be the infecting organism in this disease, seems still far from solution. Of late, much attention has been directed to the search for a filterable virus, and success has been reported by a number of independent workers. Nicolle and Lebaillly,¹ de la Rivière,² da Cunha, Magalhaes, and da Fonseca,³ and Gibson, Bowman, and Connor⁴ have reported transmission of the disease by filtrates, and von Angerer,⁵ da Cunha, Magalhaes, and da Fonseca, Leschke,⁶ Bradford, Bashford, and Wilson,⁷ and Gibson, Bowman, and Connor⁸ have cultivated minute filterable organisms.

The influenza bacillus has been relegated by many to the position of a mere secondary invader, with pneumococci and streptococci,

¹ Nicolle, C., and Lebaillly, C., *Compt. rend. Acad.*, 1918, clxvii, 607.

² de la Rivière, R. D., *Compt. rend. Acad.*, 1918, clxvii, 606.

³ da Cunha, A., Magalhaes, O., and da Fonseca, O., *Brazil-med.*, 1918, xxxii, 376, referred to in *Med. Rec.*, 1919, xcv, 457.

⁴ Gibson, H. G., Bowman, F. B., and Connor, J. I., *Brit. Med. J.*, 1918, ii, 645.

⁵ von Angerer, *Münch. med. Woch.*, 1918, ii, 1280, abstracted in *Bull. Inst. Pasteur*, 1919, xvii, 161.

⁶ Leschke, E., *Berl. klin. Woch.*, 1919, lvi, 11, referred to in *Med. Rec.*, 1919, xcv, 456.

⁷ Bradford, J. R., Bashford, E. F., and Wilson, J. A., *Brit. Med. J.*, 1919, i, 127.

⁸ Gibson, H. G., Bowman, F. B., and Connor, J. I., *Brit. Med. J.*, 1919, ii, 331.

though others, because of the frequency with which they have found it, or for other reasons, believe it to be in some way primarily concerned. If influenza is a virus disease, as now seems likely, it may be that the influenza bacillus has no primary relation to it. However, this cannot yet be asserted as a fact, for gradually the possibility is gaining recognition that familiar organisms, of supposedly fixed morphology, may assume a filterable phase. The work of Hort and Ingram⁹ and Hort¹⁰ on typhus fever, of Mathers,¹¹ Rosenow, Towne, and Wheeler,¹² Nuzum and his coworkers,¹³ and others with streptococci in poliomyelitis, of Hort and his associates¹⁴ in meningitis, and the uncompleted work of one of us¹⁵ on certain lesions probably due to fungi, all point in the same direction. Further, starting with pure cultures of ordinary bacteria, Löhnis and Smith¹⁶ have reported, though incompletely, the production of a filterable phase, possibly comparable with the filterable organisms mentioned, that showed no immediate tendency to revert to the bacterial form. As described, they seem as different from the parent organisms as the viruses are from Hort's bacillus of typhus fever, the streptococcus, the meningococcus, or Pfeiffer's *Bacillus influenzae*.

The possibility that the Pfeiffer organism may, in accordance with Hort's hypothesis, assume such a filterable phase has already been suggested.¹⁷ If so remarkable a transformation should be possible, and the virus so developed should prove to be the cause of influenza, it is important that this be recognized. For this reason special interest is attached to the question of the morphologic stability of

⁹ Hort, E. C., and Ingram, W. W., *Brit. Med. J.*, 1914, ii, 15.

¹⁰ Hort, E. C., *Brit. Med. J.*, 1915, i, 673, 826.

¹¹ Mathers, G., *J. Am. Med. Assn.*, 1916, lxvii, 1019.

¹² Rosenow, E. C., Towne, E. B., and Wheeler, G. W., *J. Am. Med. Assn.*, 1916, lxvii, 1202; *J. Med. Research*, 1917, xxxvi, 175.

¹³ Nuzum, J. W., and Herzog, M., *J. Am. Med. Assn.*, 1916, lxvii, 1205. Nuzum, J. W., *J. Am. Med. Assn.*, 1916, lxvii, 1437.

¹⁴ Hort, E. C., Lakin, C. E., and Benians, T. H. C., *Brit. Med. J.*, 1915, i, 541; *J. Roy. Army Med. Corps*, 1916, xxvi, 153. Hort, E. C., and Caulfield, A. H., *J. Roy. Army Med. Corps*, 1916, xxvii, 312. Hort, E. C., *Brit. Med. J.*, 1917, ii, 377.

¹⁵ Wade, H. W., *Philippine J. Sc., Section B*, 1918, xiii, 165.

¹⁶ Löhnis, F., and Smith, N. R., *J. Agric. Research*, 1916, vi, 675.

¹⁷ Editorial, *Brit. Med. J.*, 1918, ii, 665.

the organism. The observations to be described show that it is unstable, that the familiar bacillus is but a simple form of an organism capable of complex development. It is probably of no little significance that the most radical development seen has occurred only in association with certain bacteria, and that other bacteria have apparently the opposite, degenerative, effects. In view of the possibilities that arise from these observations, and because it will be impossible for us in the near future to extend them to the degree that seems desirable, we are led to make a preliminary report at this time.¹⁸

Our bacteriological findings during the influenza epidemic strongly indicated that the Pfeiffer bacillus plays an important part in the disease. Accordingly, we have made attempts from time to time to determine, if possible, the essential factors influencing its pathogenicity. Yanagisawa¹⁹ reported the effect of simultaneous injection of *Bacillus influenzae* and streptococci or pneumococci into white mice. Since we lacked animals necessary to duplicate the experiments, we essayed to determine the effects of cultivating these organisms together in fluid media. At the same time an attempt was made to determine whether a filterable stage might be produced in bouillons or in the synthetic media of Löhnis and Smith, with and without added blood (hemoglobin) extract. The developmental morphologic changes that appeared in certain of these first cultures were so surprising that a more extensive series of observations on this feature was made, other questions being left for future work.

Organisms Used.

The organisms used are typical strains of the influenza bacillus, obtained from autopsies. Though now relatively saprophytic in some respects, they are scarcely less exacting as to media requirements than immediately after isolation, growing not at all on non-hemoglobin agar, poorly on cool mixed (45–50°C.) blood agar, well on 58–65°C. agar, and very luxuriantly, with large, grayish colonies, on hot mixed (80–90°C.) agar. After the preliminary experiments with a single strain (Strain A) two others were chosen for parallel tests. As a

¹⁸ The more detailed report will appear in the *Philippine Journal of Science*.

¹⁹ Yanagisawa, S., *Kitasato Arch. Exp. Med.*, 1919, iii, 85.

routine precaution against possible contamination all were twice tube-seeded.

Morphologically, Strain A from blood agar tends to short plump forms, often coccobacillary, and Strains B and C to longer, rather thin, often stiff looking bacilli. However, all three may be induced to assume all the typical forms of the influenza bacillus.

The pathogenicity of these strains on subcutaneous inoculation in monkeys is now very low, much less than when first isolated. Poison production, as described by Parker,²⁰ has been determined in Strain A; the others have not been tested.

Media.

The essential media used were beef infusion bouillons made with Witte's peptone and sodium chloride in various concentrations. Synthetic media proved unsuitable. The blood extract used was made by thoroughly laking sheep or horse blood, 20 cc. per 100 cc. of distilled water, heating this to 80° or 85°C., and while hot precipitating the proteins with strong hydrochloric acid. The suspension so produced was filtered, first through gauze and then paper, and the filtrate reduced to about 1 per cent if too acid, and sterilized by filtration or repeated heating to 65°C.

Seed.

Heavy 12 to 24 hour growths on hot blood agar were used for inoculation. Material from Rothberger's neutral red agar was usually not satisfactory. The growth was removed by scraping and heavy suspensions were made in saline solution; 0.1 to 0.2 cc. of this was generally used for inoculating an ordinary tube culture. Cocci for growth in association were grown on suitable blood agar for 24 or 48 hours and suspensions made in saline solution.

Growths in Pure Fluid Cultures.

Macroscopic Growth.—Strain A has usually given heavier, more diffuse, less distinctly flocculent growths than the others. As a rule, the densities have corresponded directly to the amount of blood

²⁰ Parker, J. T., *J. Am. Med. Assn.*, 1919, lxxii, 476.

extract present and, above a certain point, inversely to the concentration of peptone and salt. In a mixture of equal parts of blood extract and normal strength bouillon growths are fairly heavy; in a similar mixture with double strength bouillon they are heavier and more diffuse. With lesser concentrations of blood extract or greater concentrations of peptone and salt they are usually progressively less.

Microscopic Growth.—Strain A has given little of the filamentous growth in these cultures; the forms developed have as a rule remained largely bacillary. In ordinary films nothing important is to be seen except an occasional element evidently branching. In Benians²¹ Congo red films these are well demonstrated (Figs. 6, 8, and 9). The filamentous tendency is seldom as evident as in Fig. 8, segmentation usually taking place promptly.

Round, spore-like bodies that structurally are comparatively delicate are also produced. Lacking the density and rigidity that preserve the morphology of ordinary bacterial elements through the process of drying and heat fixation, they are usually greatly injured in ordinary films. As a rule, they are likely to be dismissed as shrunken involution forms. They are well demonstrated in the Congo red films, where the background evidently sets before the bodies dry out sufficiently to shrink. Here they usually measure approximately 1.5 to 3 microns, often grading down to small coccoid granules, and up to a considerable size.

Conidial bodies are apparently produced in three ways: (1) by direct transformation from short bacillary elements; (2) as terminal knobs (Figs. 1 to 9) on simple rods or on short branches that might be likened to conidiophores (Fig. 9); and (3) as simple lateral buds (Figs. 2, 3, 5, and 18). Though usually single and round, even the smaller masses are not infrequently compound and lobulate (Figs. 2 and 3).

These bodies very clearly act as fungous spores, giving rise to one (Figs. 4, 5, and 8), two (Figs. 5 and 7 to 9), and sometimes even three offshoots. The offshoots are generally elongated, but sometimes develop entirely as rounded buds (Figs. 6, 7, and possibly 9). The

²¹ Benians, T. H. C., *Brit. Med. J.*, 1916, ii, 722.

bodies are therefore analogous to the conidiospores of many species of *Discomyces*.²² So far as we know, no similar structure is produced by any strictly bacterial (non-fungous) organism. There is no evidence that these elements function as spores in the bacteriological sense.

Strains B and C have exhibited at one time or another all the forms described. However, they tend to discard quickly the more bacterial forms, developing filamentous masses that often become very complex (Figs. 10 to 12). These masses are seldom more than suggested by Strain A (Figs. 5 and 6). Similar, but extremely small, closely branching and budding complexes form the chief type of growth of all strains in high concentration.

Growths in Association with Other Organisms.

Work with the organism growing in association with other bacteria has not been so extensive as with pure cultures. However, the changes seen indicate that this phase of the problem is of greater interest. Mixed directly in ordinary bouillon with a pneumococcus, rapid degeneration occurs, the bacillus completely disappearing in a few days. On the other hand, with the strains of streptococcus used in most of the experiments a remarkable development takes place. This has been traced from day to day, with all three strains, through the forms previously described to the most extreme clusters of strictly fungous growth shown in Figs. 13 to 21. Here the long, more or less frequently branching filaments and the numbers of laterally formed conidial bodies are particularly striking.

A peculiar feature is the frequent appearance of the imperfectly defined material to be seen in Fig. 20 lying between the conidial bodies. This is usually absent but may be abundant. Lobulate growth is frequently seen in large masses (Figs. 18 and 21) evidently developed from conidial bodies. These masses sometimes attain an appearance suggestive of certain as yet unpublished observations made

²² For a discussion of the validity of this term over *Streptothrix*, *Actinomyces*, *Nocardia*, etc., see, Merrill, E. D., and Wade, H. W., *Philippine J. Sc.*, 1919, xiv, 67.

by one of us in the work that led to the formulation of the "cryptoplasm" hypothesis. It would be of interest to determine whether this type of growth could progress indefinitely.

Cultivability of Described Forms.

It need not be emphasized that none of the described forms is due to involution, but result from active growth of the organism in adaptation to influences in the medium. In this adaptation the ordinary cultural characteristics are soon lost. Repeatedly, subplants on blood agar from fluid cultures have remained sterile, though it was evident that the original cultures were not dead, since they subsequently became more turbid through further growth.

There seem to be distinct stages in the depression of cultivability. Within a day or two the organism, originally growing luxuriantly on hot blood agar, produces only small, comparatively delicate growths in subcultures. A later subplant from the fluid culture may develop only minute, almost imperceptible colonies that are found to be made up of rather short, extremely fine, irregular bacilli, usually finely beaded; these are often suggestive of the leprosy bacillus; in another day the area scraped over in getting material for the original film shows a distinct haze, also made up of these fine bacilli. Subplants from this give light growths of more typical influenza bacilli, and on further subculturing the usual heavily growing type is recovered. Both processes have been observed several times.

A few attempts have been made to perpetuate the fungous growth in the fluid media, but thus far subcultures have not developed to any great extent.

DISCUSSION AND SUMMARY.

It has been found that three different strains of an organism supposed to be *Bacillus influenzae* will, under certain conditions, abandon the usual bacillary form and grow as a frank fungus, morphologically of the *Discomyces* type. Under other conditions they show less modification, the most striking feature then being the production of conidiospores, bodies of a type not found in true bacteria. That this organism may not be the true Pfeiffer bacillus is conceivable,

of course, but considering the source, morphology, ordinary cultural characteristics, and the poison production of the one strain tested, we consider this highly improbable. Further, we are confident that the cultures do not contain any contaminating organisms, as may be suggested. In short, we believe that we have been dealing solely with the true Pfeiffer bacillus.

While these observations are of considerable interest as a contribution to the biology of the bacteria of this general type it cannot, of course, be predicted that they will prove to be of any significance as regards the true causative agent of epidemic influenza. Experimental work with this organism, apparently negative so far as reproducing true clinical influenza is concerned, has been carried out with the bacillary form exclusively. It may be found that its physiological capabilities in another phase are essentially different. This is a general biological law and there is no evident reason why it should not hold true here.

It remains to be determined whether the relatively high, complex forms described have any relation to those that occur while the organism lives among other organisms on the respiratory mucosa or acts as a tissue invader. While it seems improbable that they should develop in the animal body, that is while the organism is living as a parasite, it is at least possible that the bacillus may, under some conditions, undergo some analogous or at least similarly radical modification. If this supposition is true its cultivability might well be quite different from that of its bacillary phase, in which event it might be present in abundance and yet not be found in ordinary cultures or be recognizable in films.

But the more important problem would appear to be whether it can assume a simpler phase. If, as some believe, some of the infectious bacteria and fungi can do this, whether it be as minute, filter-passing, formed elements or as a more or less amorphous ("symplastic,"¹⁶ "cryptoplasmic,"¹⁵) substance, an organism that is capable of as remarkable a range of morphological development upward might well go to the other extreme from the mean, bacillary stage. Whether or not this occurs and if so under what conditions are questions that deserve thorough investigation.

EXPLANATION OF PLATES.

Photomicrographs of unstained organisms in Benians' Congo red films.

PLATE 11.

Figs. 1 to 8 are from a 7 day pure culture of Strain A, in blood extract bouillon.
 $\times 1,000$.

FIG. 1. Formation of terminal conidial bodies; one long segmenting form; ordinary bacillary forms.

FIG. 2. One element with a lobulate terminal conidial mass.

FIG. 3. Lobulate lateral mass; terminal conidium; free conidium.

FIG. 4. Sprouting conidium; terminal conidium; ordinary bacillary forms.

FIG. 5. Two adjacent conidia, one with a single offshoot, the other with two; irregular forms.

FIG. 6. Budding conidium; irregularly branching forms.

FIG. 7. Giant conidium; small conidium with two offshoots.

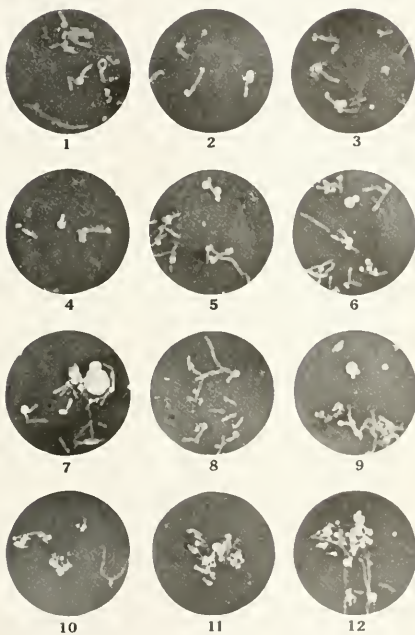
FIG. 8. Repeatedly branching growth, arising from a two-sprout conidium. Preparation for segmentation is evident.

FIG. 9. Strain A, from a 3 day culture with streptococcus, in ordinary meat infusion bouillon. Conidium with two small buds; branching form, one portion being in effect a conidiophore; associated streptococci present. $\times 1,000$.

FIGS. 10 to 12. Strain B, from a 7 day pure culture in favorable blood extract bouillon. Irregular compact growth-complexes, with short filamentous development. $\times 750$.

PLATE 12.

FIGS. 13 to 21. Strain B, from a 7 day culture in plain bouillon, in symbiosis with a streptococcus. Various stages of filamentous growth and the development of conidia. In Fig. 16 two filaments have become intertwined at one point. In Fig. 17 is seen a growth-complex on the branch from the main filament. In Fig. 18 the contrast between the mother conidium and the nearby lateral bud is striking. The mass in Fig. 20 is unusual, illustrating extreme filamentous growth with numerous conidial bodies and a peculiar indefinite growth element. Figs. 14, 15, 17, and 19 to 21, $\times 750$, Figs. 13, 16, and 18, $\times 1,000$.





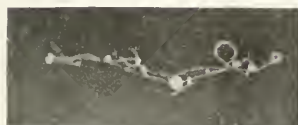
13



14



15



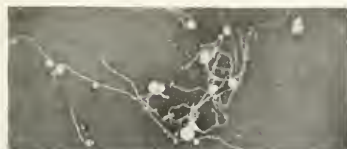
16



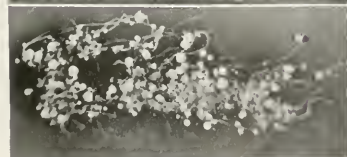
17



18



19



20



21

A METHOD OF STANDARDIZING BACTERIAL SUSPENSIONS.

By FREDERICK L. GATES, M.D.

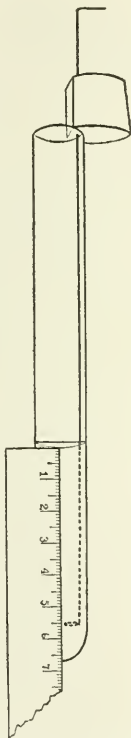
(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, October 23, 1919.)

When bacterial suspensions are used as vaccines or in serum reactions it is necessary or desirable to determine their concentration, at least approximately. Many methods have been advocated. The bacterial count per cc., the weight of the moist or dried bacterial substance, the capacity of a platinum loop, the opacity of various bacterial or chemical suspensions, the volume of the sedimented organisms, all have served as standards in various methods of determining concentration already described. The more accurate methods involve much time and labor; the simpler ones give only a rough approximation of bacterial content.

If a wire loop is gradually pushed down into a suspension of organisms in a test-tube (Text-fig. 1) and viewed by looking down into the tube through its mouth, the depth at which the loop disappears will be determined by the opacity of the supervening column of suspension. If two suspensions of the same organism are compared in this manner, a longer column of the thinner suspension will be required to effect the disappearance of the loop. The lengths of the columns may be measured and compared, and the measurements might be interpreted in terms of bacterial concentration if a suitable standard were determined. Tests with such an instrument show that with a little practice the length of the column of a bacterial suspension that will just hide the loop (the depth of disappearance) can be measured with considerable accuracy. For example, with suspensions of such an opacity that the loop disappears between 1 and 4 cm. below the surface, the zone of most accurate measurement, the depth of disappearance can be read within 1 mm. repeatedly, an error of less than 10 per cent. Within certain limits, neither the diameter of the test-tube nor the size of the wire loop affects

the readings appreciably. Even in the laborious method of counting the organisms the accepted error is often twice as great. If the relation of the depth of disappearance to the concentration could be



TEXT-FIG. 1. The simplest form of the apparatus used to measure the depth of disappearance of a wire loop in a suspension of bacteria.

determined, this would seem to be a quick and simple method of standardizing bacterial suspensions with a minimum of apparatus and manipulation.

It might be supposed that if a suspension containing 1,000 million bacteria per cc. caused the loop to disappear at 2 cm. below the meniscus and this suspension was diluted so as to contain 500 million bacteria per cc., the loop would disappear at 4 cm., or, in other words, that the opacity of a solution would vary directly with its concentration or inversely with the depth of disappearance of the wire loop. This is found not to be the case. In the second instance the loop will disappear at some distance less than 4 cm.; for example, at 2.8 or 3.3 or 3.6 cm. This discrepancy is due to the presence in each reading of a constant which must be eliminated by subtraction in order to bring the readings into ratio with the bacterial concentrations.

The constant appears to be a function of the size and opacity of the individual bacteria in the suspension. Two portions of a suspension of starch grains in cold water, one of which has been heated to boiling, give two parallel series of readings upon successive dilutions, but the constant with the heated specimen is larger than that with the unheated one, corresponding to an increase in size and translucency of the starch grains in the heated suspension. Subtraction of its own constant from each series brings the corresponding readings together and thus indicates that the heated and unheated specimens contain the same amount of starch, which is, of course, the case.

While this constant is the same for any series of readings on the same suspension, it varies with each suspension examined. The problem, therefore, is to eliminate the constant and so to bring opacity and concentration into accord. In practice this is easily done.

A series of readings taken on a suspension at successive dilutions and plotted in graphic form with the readings as ordinates and the corresponding volumes as abscissæ will be found to lie approximately in a straight line. In reality it lies along a flat curve that approaches a straight line as the suspension is further diluted. If the original concentration of the suspension is such that the loop is visible at a distance greater than 1 cm., the error introduced by assuming that successive readings fall along a straight line is not appreciable. For purposes of illustration, therefore, the straight line may be employed, as in Table I, *A*. The line is plotted in Text-fig. 2, *A—A*.

Table I, *A*, represents a series of readings on a bacterial suspension taken at dilutions obtained by adding one, two, three, and four volumes of the diluent to the original volume of the suspension. Inspection of the readings shows that they are not in the same ratios to each other as the corresponding volumes are. But if a constant quantity, in this instance 0.5, is subtracted from each reading, the remainders fall into direct ratios with the volumes, as is seen in Table I, *B*. Similar results are obtained from the graph. If the straight line is projected across the zero abscissa it crosses it at an ordinate distance of 0.5 cm. A line *B—B* parallel to *A—A* and passing through the zero point cuts each volume abscissa 0.5 cm. below the corresponding observed reading for that volume. The

TABLE I.

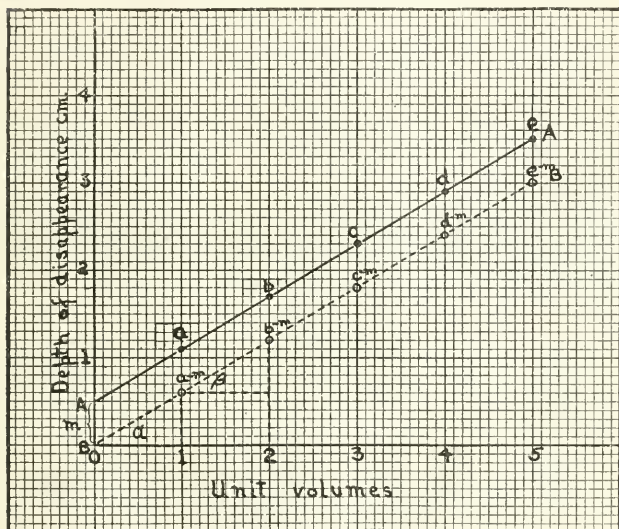
A		B	
Unit volumes.	Depth of disappearance.	Constant.	Corrected readings.
	cm.		
1	1.1	0.5	0.6
2	1.7		1.2
3	2.3		1.8
4	2.9		2.4
5	3.5		3.0

corrected reading, divided by its volume, is in each case the tangent of the angle alpha, and the corrected readings are therefore in the same ratios to each other as their corresponding volumes.

It is not necessary to plot the readings or even to find the constant in order to subtract and so to eliminate it, even though it is found to differ with each suspension examined. Each of the observed readings on a single suspension is the sum of the corrected reading and the constant. Let the successive observed readings be *a*, *b*, *c*, *d*, etc., and the constant *m*. Then the corrected readings are *a* - *m*, *b* - *m*, *c* - *m*, etc. If any corrected reading is subtracted from a subsequent one the constant is cancelled from the equation,

$$(b - m) - (a - m) = b - a \quad (d - m) - (b - m) = d - b$$

and the remainder is the difference between the observed readings. Obviously the converse is also true and the difference between any two observed readings equals the difference between the corresponding corrected readings. It is convenient to choose such dilutions of the suspension that the difference between successive readings is in



TEXT-FIG. 2. The ordinates represent the depth of disappearance in cm. of a wire loop in a suspension of bacteria. The abscissæ represent the corresponding successive dilutions of the suspension. A—A, observed readings, a , b , c , d , e . B—B, corrected readings, from which the constant m has been eliminated by subtraction. The corrected readings stand in equal proportion to the corresponding volumes,

$$\frac{a - m}{\text{vol } a} = \frac{b - m}{\text{vol } b} = \frac{c - m}{\text{vol } c} = \frac{d - m}{\text{vol } d}, \text{ etc.}$$

and may therefore be used to determine the concentration of the bacteria per cc.

each instance equal to the corrected reading for the original concentration of the suspension. This is most easily explained from the graph. Reference to Text-fig. 2 shows that since angle alpha equals angle beta, tan alpha equals tan beta, or

$$\frac{a - m}{\text{vol } a} = \frac{(b - m) - (a - m)}{\text{vol } b - \text{vol } a}$$

Then

$$a - m = \frac{\text{vol } a (b - a)}{\text{vol } b - \text{vol } a}$$

and if

$$\text{vol } b - \text{vol } a = \text{vol } a$$

then

$$a - m = b - a$$

When two observed readings are considered, the difference of whose volumes equals the original volume of the suspension, then the first, subtracted from the second, will give the corrected reading for the original volume of the suspension (Table II).

TABLE II.

Volumes.	Volume <i>a</i> .	Observed readings.	Corrected reading (<i>a</i> - <i>m</i>).
2-1 =	1	1.7-1.1 =	0.6
3-2 =	1	2.3-1.7 =	0.6
4-3 =	1	2.9-2.3 =	0.6
5-4 =	1	3.5-2.9 =	0.6

It is thus seen that the corrected reading for any suspension, by which its concentration may be compared with that of a standard suspension of the same organism, may be found by making a reading on the suspension, adding an equal amount of the diluent, making a second reading, and subtracting the first reading from the second. Any error in observation is considerably reduced, however, if the suspension is diluted several times instead of once before the second reading is made, and consideration of the equation

$$a - m = \frac{\text{vol } a (b - a)}{\text{vol } b - \text{vol } a}$$

shows that any dilution of the suspension may be used to obtain the corrected reading $a - m$. A concrete example will illustrate the point.

Suppose that in a given suspension whose volume is 2 cc. (*vol a* = 2) the loop disappears at a depth of 1.2 cm. ($a = 1.2$). The suspension is then diluted by adding, for example, 6 cc. of the diluent, so that the total volume is now 8 cc. (*vol b* = 8). Let the second reading be 3.6 cm. ($b = 3.6$). Then, substituting in the equation,

$$a - m = \frac{2(3.6 - 1.2)}{8 - 2}, \text{ or } a - m = 0.8$$

The corrected reading for the concentration of the given suspension is 0.8 cm. Now this corrected reading may be directly compared with corrected readings on other suspensions of the same organism. A suspension whose corrected reading is 1.6 cm. contains half as many organisms per cc.; one whose corrected reading is 2.4 cm. contains one-third as many. In this connection it should be remembered that the value obtained for $a - m$ applies to the original suspension before the dilution required in the test. If the diluted test specimen is to be used for any purpose its corrected reading may be obtained from the equation,

$$b - m = \frac{\text{vol } b (b - a)}{\text{vol } b - \text{vol } a}$$

If several suspensions of the same organism are simply to be compared with one another no further calculations are necessary. In many instances, however, a permanent standard is desirable and it is convenient to translate the opacity into terms of an accepted standard, such as the weight of dried bacterial substance or the number of organisms per cc. The bacterial count is the basis most widely employed, even though it is recognized that similar counts do not always represent equal amounts of bacterial substance, on account of variations in the size of the organisms under varying conditions of cultivation.

Given a corrected reading on the depth of disappearance of a wire loop in a suspension of a microorganism and the corresponding bacterial count, or better, a series of such correlated observations, to reduce the error in counting as well as in estimating opacity, the depth of disappearance of a standard suspension containing, for example, 1,000 million bacteria per cc. is readily found by inverse proportion.

$$\frac{\text{Opacity of standard suspension (unknown)}}{\text{Opacity of given suspension (in cm.)}} = \frac{\text{Count on given suspension (millions per cc.)}}{\text{Count on standard suspension (1,000 millions)}}$$

The opacity (depth of disappearance) of a standard suspension of 1,000 million bacteria of any species having been thus determined, the number of organisms per cc. in any suspension of such bacteria may be readily calculated from the same equation. A complete example follows.

Suppose that the readings quoted on page 111 were made on a suspension of normal type meningococci. It has been found that 1,000 million normal meningococci correspond to a depth of disappearance of 4.2 cm. In 2 cc. of the given suspension the loop disappears at 1.2 cm. When this suspension is diluted to 8 cc. the loop disappears at 3.6 cm. The corrected reading for this suspension is then

$$\frac{2(3.6 - 1.2)}{8 - 2}, \text{ or } 0.8 \text{ cm.}$$

which, substituted in the equation above, gives

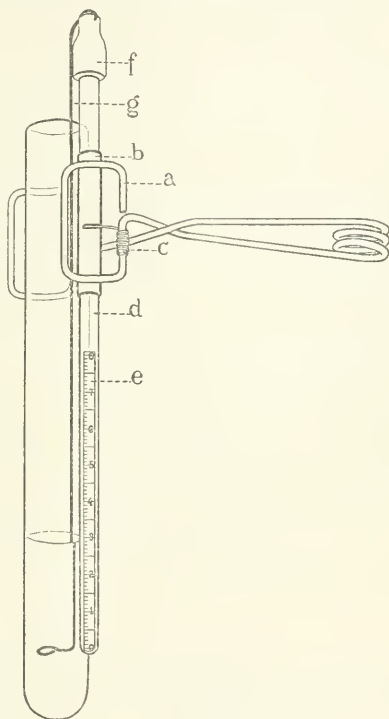
$$\frac{4.2}{0.8} = \frac{x}{1,000}$$

The suspension under examination contains 5,250 million meningococci per cc.

Several simple forms of apparatus have been used to measure the depth of disappearance. The first observations were made with a loop of No. 18 nichrome wire thrust through a cork, as in Text-fig. 1, and measured with a centimeter scale laid against the test-tube. A more convenient instrument, which has proved sufficiently accurate for ordinary use, is shown in Text-fig. 3.

To a wire test-tube clamp (*a*) is soldered a 4 cm. piece of 7 mm. (inside diameter) brass or copper tubing (*b*) slotted in the middle to receive the free end of a small coiled spring (*c*) which presses against an 18 cm. length of glass tubing (*d*), holding the tubing in place, but allowing it to be raised or lowered freely in the tube. A narrow paper centimeter scale (*e*), reading upward, is sealed within the glass tubing, which is surmounted by a stub of heavy walled rubber tube (*f*) to hold the end of the No. 18 gauge nichrome wire loop (*g*). Iron wire may be used, but it rusts and flakes off when repeatedly wet and heated. Nichrome or chromel wire retains its black color and is unaffected by repeated use. The free end of the wire is bent at right angles into a small circle, so that it lies horizontally in the center of the test-tube opposite the zero point on the centimeter scale when the instrument is held in the upright position.

In use a measured quantity of the specimen to be estimated is placed in a sterile test-tube, 1.6 by 16 cm., in the clamp. The wire loop, viewed by looking down into the mouth of the test-tube, is



TEXT-FIG. 3. A convenient modification of the instrument for measuring the opacity of bacterial suspensions.

lowered into the suspension and adjusted until it is just beyond the limit of vision through the fluid; *i.e.*, the opacity of the supervening column of suspension is just sufficient to hide the loop. This

end-point is more accurately observed than one with the loop faintly visible. The depth of disappearance is then read on the centimeter scale at the bottom of the meniscus, care being taken that the test-tube is held perpendicularly, with the meniscus at the level of the eye. A measured amount of the diluent is then added and mixed by agitation, and the second reading is made. The original volume of the suspension (*vol a*), the amount of diluent added (*vol b - vol a*), and the two observed readings (*a* and *b*) give the necessary data for obtaining the corrected reading (*a - m*) on the suspension. This corrected reading, by comparison with the standard for the given organism, figured by inverse proportion as already demonstrated, gives the concentration of the suspension in millions of organisms per cc. A separate sterile test-tube should be used for each suspension examined. The nichrome wire loop is dried and sterilized in a flame. The rubber cap (*f*) permits it to be held out at a right angle for this purpose.

The readings and the calculations, on a slide rule, can be made in 2 or 3 minutes when the standard for the given organism is known. Owing to differences in acuity of vision, a certain personal equation is involved in the reading of the end-point, and the standards should be worked out for each observer by comparison of corrected depth of disappearance readings and the corresponding bacterial count. Once the standards are established, suspensions of the same organism can be estimated rapidly. The method should be found useful in vaccine and serological laboratories in which many suspensions have to be standardized.

SUMMARY.

The opacity of a bacterial suspension is measured by the length of the column of the suspension required to cause the disappearance of a wire loop. By a simple formula the measured opacity is translated into terms of the concentration of bacteria per cc., and so made comparable with that of other suspensions of the same organism. An instrument for measuring the opacity of bacterial suspensions is described in detail.

MYCOSIS OF THE BOVINE FETAL MEMBRANES DUE TO A MOULD OF THE GENUS MUCOR.

By THEOBALD SMITH, M.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical
Research, Princeton, N. J.)

(Received for publication, December 6, 1919.)

An accurate knowledge of the number and variety of living organisms which may invade the uterochorionic space during pregnancy in bovines and multiply enough to set up localized or general disease of the chorion can only be gained by a cumulative study of the pregnant uterus before discharge of the fetus. After expulsion of the latter, adhesion of the placenta, the loss of pathological fluids held between uterine wall and chorion, the soiling of the placenta by the bedding, or its destruction by the cow, all stand in the way of an elucidation of those still unknown agencies of disease which may be of far more significance than is credited to them. The relation of *Vibrio fetus* to abortion may be considered as established. Occasionally *Bacillus pyogenes* is present in such numbers in the organs of the discharged fetus that tentatively it may be regarded as a primary agent in certain cases.¹

Among a considerable number of pregnant uteruses obtained from abattoirs, the writer found one which deserves a brief description. The uterus was brought intact to the laboratory and the following facts were ascertained.

Case 182.—When the wall of the uterus was cut through there was found between uterine mucosa and chorion (uterochorionic space) a considerable amount of a turbid fluid full of small flakes. Over a region of the chorion about 20 cm. in diameter, corresponding with or resting on the left shoulder and thorax of the fetus, the cotyledons, five in number, are separated from the uterine wall so that the latter

¹ Smith, T., *J. Exp. Med.*, 1919, xxx, 325. Zwick and Zeller, *Arch. k. Gsndhtsamt.*, 1913, xliii, 1.

can be lifted away from the fetal membranes over this area. The pedicles of these cotyledons (maternal caruncles) are short, blunt projections of the uterine wall, varying in size, in color grayish with some hemorrhagic spots. The affected cotyledons are enlarged, the margins much thickened, and rolling cup-shaped over into the central necrotic portion. The chorion between these detached cotyledons is beset with yellowish gray, slightly elevated plaques, 2 to 4 mm. in diameter and a fraction of a mm. thick, producing a coarse goose-skin appearance. The subchorionic edematous tissue appears as a glassy, gelatinous layer 1 to 2 cm. thick. In one horn the chorion, over an area 6 cm. square, is beset with grayish to yellowish plaques containing minute hemorrhages. The fluid in the allantois at both horns is clear. The amniotic fluid contains a fairly abundant amount of fecal matter. The cervix of the uterus is normal and the external os is tightly closed and provided with the normal mucous plug.

The fetus is 25 inches long and normal, with the following exceptions. There is edema of the sheath of the umbilical vein within the abdomen, also slight edema of the capsule of the kidneys, and of the interlobular tissue of the lungs. The rumen contains considerable yellowish (fecal) matter, but the fourth stomach contents are still clear and colorless.

Although the gross appearances did not correspond closely to those found in the presence of *Bacillus abortus*, the case was considered one of infectious abortion due to this bacterium, until examination of the scrapings of the cotyledons showed a branched mycelium and no bacteria of any description. Cultures on slanted agar, sealed with sealing-wax and unsealed, of material from the surface of the chorion and from the amniotic fluid developed a mould. Cultures from the lungs and meconium of the fetus also contained only a mould, while those from the fourth stomach, spleen, liver, and one kidney remained sterile. Subsequent study of the moulds from placental and fetal cultures showed them to be identical and belonging to the genus *Mucor*.

The absence of *Bacillus abortus* from the cultures was confirmed by the results of inoculation of guinea pigs with the fluid exudate in the uterochorionic space, with amniotic fluid, and with contents of the fourth stomach of the fetus. The guinea pigs killed after 26 days

were normal and cultures remained sterile. Films from the chorion and the fluid exudate showed no bacteria of any kind. In the necrotic masses scraped from the cotyledons, a branched, non-septate mycelium was detected.

Tissues fixed in Zenker's fluid from various diseased and normal regions of the placenta were studied in sections in order to determine the relation of the mould to the tissues and the character of the tissue reaction. Although a variety of dyes was used, the mycelium stained very poorly and only careful focusing with a partly closed condenser made it possible to identify the filaments and trace them as far as the section permitted.

The remnants of the caruncles projecting from the uterine wall were densely infiltrated and covered with masses of polynuclear leucocytes. Among these masses the branched mycelium could be traced, penetrating them in various directions. The corresponding cotyledons with the sloughed off portions of the caruncles still embedded in them presented a variety of changes, consisting chiefly of necrosis of most of the tissue and masses of polynuclear leucocytes. Traces of the original tissue appeared in islands of villi, densely filled with blood corpuscles. Branched mycelium could be seen penetrating the necrotic and purulent foci. It varied somewhat in diameter and was more or less angular and dilated in places. Most of the filaments appeared empty, at least the staining did not reveal any contents. No fruiting bodies, either in the form of sporangia or chlamydospores, were found.

As stated above, the intercotyledonous areas of the affected region of the chorion were beset with hemispherical elevations about 2 to 4 mm. in diameter, which gave the membrane a coarse goose-skin appearance. The sections showed that over the elevations it was deprived of its epithelium and the subjacent, bared zone filled with dense masses of nuclear debris which projected somewhat and gave the surface its nodular outline. This lesion is similar to that found in the bared chorion when *Bacillus abortus* is the agent, as well as in cases associated with *Vibrio fetus*. It would seem as if any injury to or destruction of the epithelium leads to a movement of leucocytes towards the surface, under which they gather in dense groups and disintegrate.

The invasion of the fetal membranes by a mould presupposes some primary focus in the body of the dam, whence spores might enter the circulation and break through into the fetal cotyledons. Unfortunately nothing is known of the dam, since the cow was slaughtered in the routine work of the abattoir and only the pregnant uterus reserved. If the above view of the source of the infection is correct it would imply the existence of moulds in the lungs of the cow where the oxygen requirements are sufficient for the production of sporangia and spores.

The literature on pathogenic mucors is very meager as contrasted with that on *Aspergillus*. Lichtheim² isolated from moistened bread two species pathogenic for rabbits after intravenous injection of fairly large doses of spores, *Mucor rhizopodiformis* and *Mucor corymbifer*. The chief loci of germination and growth were the kidneys, the lymphoid tissue of the intestines, and the mesenteric nodes. Paltauf³ soon afterwards found a mucor in focal lesions of the cerebrum, cerebellum, lungs, pharynx, and ileum of a man 52 years old. The mucor was not cultured. The dimensions given indicate that he had a much smaller type under observation than the types mentioned above. Lindt⁴ isolated two additional species from moistened, incubated wheat bread which were pathogenic for rabbits after intravenous injection of spores. These were named *Mucor pusillus* and *Mucor ramosus*, both distinguishable from one another and from the species cultured by Lichtheim on account of the shape and dimensions of the spores. Hückel⁵ isolated a mucor from a cerumen plug taken from the ear of a patient 28 years old. This he identified with *Mucor corymbifer*.

Biological and Pathogenic Characters.

The mould was readily cultivated at incubator temperature on ordinary nutrient agar, although an addition of 1 per cent dextrose increased the vigor of the growth. At 70°F. the growth was much

² Lichtheim, L., *Z. klin. Med.*, 1884, vii, 140.

³ Paltauf, A., *Virchows Arch. path. Anat.*, 1885, cii, 543.

⁴ Lindt, W., *Arch. exp. Path. u. Pharmacol.*, 1886, xxi, 269.

⁵ Hückel, A., *Beitr. path. Anat. u. Physiol.*, 1886, i, 117.

slower. It presented the following characters. A dense, branching, silken, whitish mycelium spreads over the surface and, in tubes, penetrates vertically for a distance of 5 to 10 mm. into the depths of the agar. The mycelium varies from 4 to 10μ in diameter. It contains a slightly refractile, coarsely or finely granular cytoplasm. The branching of the mycelium was at no time observed to follow any definite scheme. It evidently depended on the condition of the substrate.

In addition to this adherent layer of interlacing filaments an abundant aerial woolly mass of filaments appeared within 48 hours. These were found empty and often collapsed later on, probably as a result of drying. Rhizoids, or radiations of short filaments from centers of growth on the agar surface, were found, from which fruiting hyphæ arise to bear at the free end the sporangia. These sporangiophores and the rhizoids can be distinguished from the vegetative mycelium by their brownish tint under a low power. The rhizoid gives origin to a short trunk which soon divides irregularly into a small number of sporangiophores.

The sporangia, barely visible to the unaided eye, become a grayish brown color as they ripen and appear almost black under a low power in transmitted light. They are subspherical, measuring transversely about 80μ and vertically about 64μ , although smaller heads occur. The columella seen after discharge of the spores projects as a spherical body from the funnel-shaped, expanding end of the supporting hypha. The slightly brownish spores are spherical and measure about 4.5μ , although some as small as 4μ and as large as 5μ may be seen.

The mycelium on the agar surface may form in places two transverse septa close together. The intervening walls of the filaments bulge and the segment thus isolated and containing a granular cytoplasm forms a rather thick wall showing later on a double contour. The filaments on both sides of this new body shrivel. In rare instances a series of such segregated and encysted masses of cytoplasm may form. These bodies, usually denominated chlamydospores (*Gemmen*, *Dauerzellen*), were quite numerous in all cultures. No zygosporos were seen at any time. An examination of the literature indicates that Lichtheim's *Mucor rhizopodiformis*² comes nearest to

the species under investigation, although there are certain differences such as a colorless, slightly larger spore (5 to 6μ) in Lichtheim's culture.

No special studies were made upon the behavior of this species under different environmental conditions. This much was frequently observed, however, that the mould is very capricious in the production of sporangia. Apparently the same conditions at one time lead to a rapid, copious formation of sporangia with ripening of spores in 2 to 3 days, in another to none. The obvious interpretation is that slight unrecognized differences in the environment exercise a decisive influence. Evidently oxygen is an important factor, for the sealing of tubes, which reduces the oxygen tension through absorption of the oxygen by the culture medium and by the mould during the early stages of growth, interferes with spore production.

To determine whether spores of the mould would germinate and develop a mycelium in the organs of rabbits, the growth from a dextrose agar culture was thoroughly stirred about in sterile bouillon and filtered through five layers of sterile fine bandage material to remove fragments of mycelium and clumps of spores. The resulting feebly clouded, lightly brownish fluid was found with only isolated spores. Doses of 1.5 and 0.5 cc. were injected into an ear vein of two rabbits weighing 2,218 and 2,404 gm. respectively. The rabbit receiving the smaller dose was in appearance normal until the 8th day, when it was chloroformed. The weight had dropped from 2,404 to 2,286 gm. The spleen was a trifle large and congested. It was beset with numerous subcapsular, 0.5 mm., isolated or agglomerated, whitish, slightly projecting nodules. Both kidneys show the same kind of nodules, about 1 to 5 mm. apart. Some of these correspond to opaque whitish streaks passing through the medulla and continuing in slenderer radial lines to the pelvis. In the latter no changes are evident. Scattering nodules occur in the liver and one in a Peyer's patch. The appendix is free. Several mesenteric nodes contain large necrotic foci. In one kidney nodule, crushed between slides, branching mycelium could be traced for some distance. Bits of spleen, liver, and kidney tissue placed in agar slants developed a rich mycelium within 24 hours.

The second rabbit died unexpectedly on the 7th day. No disturbance of health had been noticed but the weight at death was only 1,740 gm. Lesions much smaller than in the first rabbit were found in spleen and kidneys. The spleen was small and pale. The cause of death was probably some intercurrent influenza, since the lungs were generally congested and edematous and the nostrils ringed with dry crusts. There was, however, no similar disease among the older stock animals and it may be that the resistance had been lowered by the mould.

Sections of tissue of the first rabbit fixed in Zenker's fluid presented a number of interesting details which can only be briefly enumerated. The lesions of the kidneys in the cortex were made up of dense collections of polynuclear leucocytes within areas in which the interstitial cells had also proliferated. The affected area included one or several glomeruli and the tubules immediately surrounding them. The origin of the lesion, whether in a glomerulus or the tubules, could no longer be determined, as the original structures were barely identifiable. The focus thus begun could be traced downward, the associated tubules being filled with masses of partly disintegrated cells, chiefly polynuclears. These plugs extend to the tip of the papilla. Here the pelvis contains a mass of polynuclears completely surrounding the papilla. Branched mycelium could be seen in all the involved tubules. It was most abundant in the cellular masses of the pelvis. The changes in the spleen were similar to those in the cortex of the kidney. In the mesenteric nodes the foci were so numerous as to coalesce and involve more than half the node. Mycelium was specially abundant. In the liver two kinds of lesions were present, collections of polynuclear leucocytes taking the place of destroyed liver tissue and much smaller foci composed of multinucleated cells, some closely resembling those of the tubercle. No formed elements could be made out within these giant cells, but it is probable that they contained mould spores. Microscopic foci were not found in the lungs although all the injected spores must have passed through these organs.

These two inoculations serve to place this mucor among Lichtheim's so called pathogenic species. There are certain minor differences between his results and the present, which might disappear in

larger series. The slightly lower pathogenic effect when compared with Lichtheim's tests may be due to the fact that the inoculations were made after the mould had been kept 23 months on culture media.

The source of the infection in the cow can only be conjectured. The two widely distributed mucors, not pathogenic for rabbits, *Mucor mucedo* and *Mucor racemosus*, occur frequently in dairy products,⁶ and it is probable that the species under consideration may be found there and thus represent a possible source of the infection.

SUMMARY.

A mucor, closely resembling Lichtheim's *Mucor rhizopodiformis*, was isolated from the diseased chorion of a cow and from the lungs and digestive tract of the fetus. No other microorganisms were detected. The mucor was demonstrated in teased preparations from the fresh cotyledons as well as in sections of tissues suitably hardened. It produced focal lesions in rabbits following the intravenous injection of spores. The condition of the amniotic fluid and the contents of the rumen of the fetus justify the inference that premature expulsion was impending.

⁶ Weigmann, H., in Sommerfeld, P., Handbuch der Milchkunde, Wiesbaden, 1909, 328.

EXPERIMENTS ON THE NASAL ROUTE OF INFECTION IN POLIOMYELITIS.

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In this paper we shall describe experiments bearing on the intranasal route of infection in poliomyelitis. That the inciting microorganism or virus of poliomyelitis enters the central nervous system by way of the nasal passages is now generally believed. Moreover, it has been shown that this virus may be present on the nasal mucosa without inducing any signs of disease.

Both healthy and so called chronic carriers of the virus of poliomyelitis occur in man. Wide diversity of view prevails as to the frequency with which carriage of the virus arises and as to the period of persistence of the virus in the carriers. According to one group of observers (Wickman,¹ and Kling, Pettersson, and Wernstedt²), healthy and chronic carriers arise numerous during epidemics of poliomyelitis and actually exceed, possibly even many fold, the number of actual cases of the disease. Moreover, the virus may be very persistent in carriers who have recovered from an attack of the disease and be detectable by animal inoculation several months after all the acute symptoms have subsided (Kling, Pettersson, and Wernstedt). However, it should be remarked here that the virus is supposed to undergo gradual deterioration and thus fail in producing typical experimental poliomyelitis, although it is still capable of exciting atypical symptoms and lesions.

Another group of experimenters has come to quite opposite conclusions. Thus Flexner and Amoss³ who employed excised tonsillar and adenoid tissue for inoculation did not find either the great frequency of occurrence or the long survival of the virus in convalescents implied in the preceding statements. On the contrary, while they found the tonsillar and other tissues infective for monkeys during the early period of the disease in man, they observed no effects, as a rule, from the inoculation of the tissues taken after the acute symptoms had subsided.

¹ Wickman, I., Beiträge zur Kenntnis der Heine-Medinschen Krankheit, Berlin, 1907.

² Kling, C., Pettersson, A., and Wernstedt, W., *Communications Inst. méd. État Stockholm*, 1912, iii, 5.

³ Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1919, xxix, 379.

The point of difference involved is important, not only on the general basis of public health considerations, but also because of its bearing on our conceptions concerning the epidemiology of poliomyelitis. The morbidity of poliomyelitis even during severe epidemics is low. This circumstance has been accounted for by assuming either that a relative insusceptibility to the disease exists among general populations, which is no explanation at all, or that because of the wide dissemination of the virus during an epidemic and even in interepidemic periods, an unperceived active immunization of the community takes place. Insusceptibility then is due to specific protection or immunity.

In all instances in man and monkey in which an experimental inquiry has been made, it has been found that when active immunity exists, the blood carries neutralizing or destructive bodies for the poliomyelitic virus. No systematic study of the blood of exposed persons who have remained free from obvious poliomyelitis has been made. We conducted a number of tests of the blood of nurses, doctors, and others who had been repeatedly exposed during the severe epidemic in New York State in 1916, without, however, obtaining any clear and decisive results.

The kind of assumed protection just indicated would be general and specific. But experiments of Amoss and Taylor⁴ have shown that another kind of potential protective mechanism is demonstrable in man. This device is local and depends upon the presence in the nasal membrane and its secretions of a substance, not yet defined, which possesses the power of neutralizing or otherwise destroying the virus of poliomyelitis. It is suggested that this local process may play an important part in determining the morbidity of epidemic poliomyelitis.

The experiments to be described in this paper relate to several aspects of the problem of intranasal infection in poliomyelitis and bear, therefore, on the preceding discussion. The first experiments to be given concern the question of the power of the nasal mucosa of the monkey to suppress the virus of poliomyelitis directly applied to it; or, in other words, the ascertaining of the period of survival of the virus on the nasal membrane.

⁴ Amoss, H. L., and Taylor, E., *J. Exp. Med.*, 1917, xxv, 507.

Fate of the Virus Applied to the Nasal Mucosa.

An effective means of inducing infection in *Macacus* monkeys is to apply an active poliomyelitis virus to the nasal membrane on a cotton pledget. By virus in this connection is meant the comminuted spinal cord and medulla of a monkey which has suffered from acute experimental poliomyelitis.

Not all monkeys so treated acquire infection; indeed, the percentage of successful inoculations by the nasal route is smaller than by the brain or intracerebral route. The first question to present itself, therefore, is the fate of the virus in animals which may not succumb to intranasal inoculation.

It has already been shown that given an active virus, it can be detected in the nasal membrane by means of subinoculation. That is, if the nasal membrane carrying the virus is excised, ground with sterile sand, suspended in isotonic saline solution, and filtered through a Berkefeld candle, the filtrate will set up poliomyelitis in another monkey into which it is injected intracerebrally and intraperitoneally. The experiments to follow show that with a constant sample of the virus and a uniform mode of inoculation, the survival of the virus on the nasal membrane is irregular and individual.

The virus was one which has long been kept active by monkey passages, and the mode of application was by cotton pledget, which was allowed to remain in a naris for 2 or more hours. Upon removal, the animals were kept under close observation for varying lengths of time, their condition was noted, and the excised nasal membrane, after etherization of the selected animals, employed for obtaining the filtrate, as described above, for purposes of inoculating other monkeys.

Experiment 1.—May 25. *Macacus rhesus* A. Cotton pledget carrying the virus remained in a naris over night. 60 hours after the removal of the plug, the animal was killed with ether and the nasal mucosa excised. The filtrate prepared from this membrane was inoculated into *Macacus rhesus* B.

May 28. *Macacus rhesus* B. Received 2 cc. of the filtrate by intracerebral and 5 cc. by intraperitoneal injection. This animal remained well until June 3, when it showed excitement and an ataxic, uncertain gait. The symptoms extended rapidly, paralysis occurred, and the animal died on June 6. The lesions present in the spinal cord and medulla were typical of poliomyelitis.

This experiment shows, therefore, that it is possible to detect the virus by the methods employed at least 60 hours after its application to the nasal membrane. But other tests carried out simultaneously or subsequently on other monkeys killed at the expiration of 40 and 60 hours and 8 days after the removal of the pledget resulted negatively. Hence this experiment may be taken to indicate that the nasal mucous membrane of the *Macacus rhesus* possesses in some instances striking power of destroying or eliminating the virus of poliomyelitis energetically applied to it.

The property of the nasal mucosa to render ineffective, under certain circumstances, an otherwise efficient dose of the virus may be shown in still another way.

The manner of invasion of the central nervous system by the virus of poliomyelitis is still an open question. In view of the difficulties surrounding experimental infection by way of the blood, and the relative ease with which it is accomplished by way of the brain, nasal membrane, sciatic nerve, peritoneum, eye, and even subcutaneous tissue, Flexner suggested that in all instances the passage of the virus from the periphery to the center is ultimately by way of the nerves. According to this view, the virus applied to the nasal mucosa extends along the short olfactory nerve fibers to the brain and spinal cord. A certain amount of support for this mode of infection is supplied by the experiments of Landsteiner and Levaditi⁵ and of Flexner and Clark,⁶ in which after an intranasal inoculation the brain and cord of the monkeys were removed before any symptoms appeared and injected separately into other monkeys. Flexner and Clark noted that 48 hours after an intranasal inoculation the olfactory lobes but not the medulla and spinal cord might be infectious.

But illuminating as this experimental result is, it must be regarded as the exception rather than the rule. It happens also and perhaps much more frequently that after an intranasal inoculation the virus cannot be detected either in the mucous membrane or in any portion of the central nervous system. The following protocol illustrates this point.

⁵ Landsteiner, K., and Levaditi, C., *Ann. Inst. Pasteur*, 1910, xxiv, 833.

⁶ Flexner, S., and Clark, P. F., *Proc. Soc. Exp. Biol. and Med.*, 1912-13, x, 1.

Experiment 2.—Macacus rhesus. June 1. The cotton pledget carrying the active virus was permitted to remain in the naris for 24 hours. No symptoms had developed by June 5, when the animal was etherized, 88 hours after the tampon had been removed. The right middle turbinate, at the site of the tampon, showed a small hemorrhage into the mucous membrane.

The nasal mucosa and heavy suspensions of the olfactory lobes, postrolandic convolutions, medulla, and cervical and lumbar spinal cord were inoculated separately into the brains of other *rhesus* monkeys. In no instance was infection secured. The control *Macacus rhesus* in which the pledget remained for the same period became paralyzed on the 7th and died on the 9th day. The lesions of the central nervous organs were typical of poliomyelitis.

The virus used in this experiment was active and the procedure adequate. The difference in the results may be attributed to the power of the nasal mucosa in the one and not in the other animal to destroy the virus. This is the more probable explanation, although it is, of course, possible that at the expiration of the 88 hour period the increase of the virus was insufficient to flood the central nervous system so as to be detectable by the inoculation test.

The first view given is, however, supported by another experiment in which the cotton plug carrying the active virus was permitted to remain in the naris only 2 hours. One of the *Macacus rhesus* monkeys developed symptoms, became paralyzed, and the nervous organs showed typical lesions of poliomyelitis. The other showed no symptoms and was etherized on the 16th day. The nasal mucosa, olfactory lobes, and medulla were injected intracerebrally into three *rhesus* monkeys of which none developed symptoms.

Effects of Antiseptics.

The innate destructive property possessed by the nasal membrane for the virus of poliomyelitis may be regarded as a valuable defensive mechanism. The question has often been raised whether, during an epidemic of poliomyelitis, the application of antiseptics to the nasal mucosa is to be recommended. In the case of chronic meningococcus carriers, the suppression of that microorganism by the introduction of antiseptics directly into the nasopharynx has not been notably successful; and the meningococcus is apparently a much more fragile organism than the microbe of poliomyelitis.

There is a further important consideration. Now that it has been shown that the nasal membranes are themselves defensive, account needs to be taken of the action of antiseptic drugs upon the chemical substances in the membranes upon which their protective function depends. It is fortunate that in the case of poliomyelitis the effects of chemical antiseptics on the virus of poliomyelitis implanted on the nasal mucosa can be directly tested experimentally.

We already possess fair data of the effects of disinfectants on the virus *in vitro*. The effective chemicals chiefly studied are hydrogen peroxide (Flexner and Lewis), formaldehyde (Römer), thymol, potassium permanganate (Landsteiner and Levaditi), and still others. In the experiments to be given the only antiseptics employed were chloramine-T and the oily solution of dichloramine-T, as devised by Dakin and Dunham.^{7, 8}

Two protocols only of this series of experiments will be given. In a few instances in which the virus was applied to the nasal mucous membranes, monkeys treated with the dichloramine-T did not become ill or paralyzed, but as in these cases the control animals also failed to come down, it was considered probable that the particular sample of the virus employed for inoculation was ineffective, or that all the animals used were refractory.

Experiment 3.—Macacus rhesus. Apr. 16, 5 p.m. Inserted tampon with virus into left naris. Apr. 17, 10 a.m. Removed tampon which was slightly blood-stained. Both nares washed with 1:1,000 chloramine-T solution in water, after which the dichloramine-T in oil was sprayed into the nostrils; twenty-five successive expulsions of the oily solution by hand pressure were made for each side. The spraying was repeated at 12 m. and 2, 4, and 6 p.m. Apr. 18. Spray every 2 hours from 8 a.m. until 6 p.m. Apr. 23. No symptoms had appeared until this date on which the animal showed excitement and paralysis of the left arm. Apr. 25. Animal generally paralyzed and prostrate. Apr. 27. Animal dying; etherized. The lesions in the central nervous organs were typical of poliomyelitis.

⁷ Dakin, H. D., and Dunham, E. K., A handbook on antiseptics, New York, 1917, 33.

⁸ We are indebted to Dr. E. K. Dunham for the preparation used in our experiments and for advice as to the procedure to follow.

The conditions of the above experiment are severe. The tampon was left in the naris for 17 hours, and injury, as indicated by the blood staining, had been inflicted on the mucous membrane. In the next experiment the conditions are less severe, but the result was not essentially different.

Experiment 4.—Macacus rhesus. May 25. Oily dichloramine-T solution sprayed into nares at 2, 4, and 6 p.m. May 26, 8 a.m. Spray as before. 10 a.m. Inserted cotton plug carrying the virus into the left naris. 12 m. Removed tampon and applied the oily spray. Repeated the spray at 2, 4, and 6 p.m. May 29. Animal protects the left leg. June 3. Ataxic; excited. June 4. Extensive paralysis; prostrate. June 8. Dead. The spinal cord and medulla showed typical lesions of poliomyelitis.

The two experiments given do not, of course, show conclusively that the application of antiseptic fluids to the nasopharynx exercises no restraining influence on the multiplication and pathogenic action of the virus of poliomyelitis present there. The conditions of the experiments may well have been too severe to be readily comparable with those arising in man. But account must also be taken of the fact that monkeys sometimes resist the introduction of the virus by means of tampons without any aid to the defensive powers of the nasal membranes whatsoever. If so active an antiseptic agent as the chloramines may thus be ineffective, it would seem that even less could be expected of the indiscriminate chemical solutions often applied by sprays to the nasopharynx.

Blocking Infection via the Nasal Mucosa.

The mere plugging of the naris of a *Macacus rhesus* with a tampon carrying the active virus of poliomyelitis may not suffice to set up infection. The outcome is determined not only by the degree of activity of the sample of the virus, but also by the strength of the defensive mechanisms possessed by the particular animal. It has been shown that the nasal mucosa is protective, but it appears also that other and deeper mechanisms play a part in supporting or reinforcing the nasal defenses.

One of the deeper mechanisms is the meningeal-choroidal plexus complex, as pointed out by Flexner and Amoss.⁹ The latter ascertained that an otherwise ineffective virus tampon could be rendered effective if the integrity of this complex was disturbed as, for example, by setting up within it a temporary chemical inflammation. Various mild chemical irritants suffice for this purpose, but sterile alien serum is highly satisfactory.⁹ But the particular point which the next experiments illustrate is not so much the fact of the promotion of nasal infection by the method indicated as the means employed to block infection by way of the nares.

Flexner and Amoss^{9,10} have shown also that the introduction of immune poliomyelitic serum by lumbar puncture into the subarachnoid space in monkeys suffices to prevent infection by way of the meninges, blood, naris, etc. The question which was now investigated was whether blocking of the nasal infection could be secured by means of the immune serum injected into the blood.

The first protocol given is that of an unsuccessful attempt to block infection by way of the nares by means of hexamethyleneamine. This drug does display some power of destroying or of inhibiting the development of the virus of poliomyelitis *in vivo* (Flexner and Clark¹¹). But its inferiority to immune serum is great, and the experiment which follows can be viewed in that light and also as another control observation.

Experiment 5.—Macacus rhesus. Mar. 6. 2 cc. of sterile normal horse serum injected intraspinally. Mar. 7. 2 hour virus-carrying cotton tampon in naris. Mar. 9. 0.5 gm. of hexamethyleneamine in 10 cc. of water given by stomach tube twice a day. Treatment repeated daily for 6 days. Mar. 15. No symptoms. Mar. 16. Animal excited, somewhat ataxic, and protects the right arm. Mar. 22. Condition remained stationary until this date, when the paralysis involved both arms, back, and right leg. Mar. 28. Dead. The lesions present in the central nervous organs were typical of poliomyelitis.

Experiment 6. (a) Control.—Macacus rhesus. Feb. 8, 4 p.m. 2 cc. of normal horse serum injected intraspinally. Feb. 9. 2 hour nasal plug carrying the virus. Feb. 18. Arms paralyzed; back and right leg weak. Feb. 19. Pros-

⁹ Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1917, xxv, 525.

¹⁰ Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1914, xx, 249.

¹¹ Flexner, S., and Clark, P. F., *J. Am. Med. Assn.*, 1911, lvi, 585.

trate. Feb. 20. Etherized. The medulla and spinal cord showed typical lesions of poliomyelitis.

(b) *Test*.—*Macacus rhesus*. Feb. 8, 4 p.m. 2 cc. of normal horse serum intraspinally. Feb. 9. 2 hour nasal plug carrying the virus. On removal the plug was slightly blood-stained. Feb. 12. 5 cc. of immune monkey serum, obtained by pooling the blood from several animals, injected intravenously. This animal was kept under close observation for 2 months during which time no symptoms arose.

Experiment 7. (a) Control.—*Macacus rhesus*. May 14. 2 cc. of normal horse serum injected intraspinally. May 15. 2 hour virus-carrying plug in naris. May 16. 2 cc. of normal horse serum intraspinally. May 17. Repeated 2 hour nasal plug. May 18. 2 cc. of normal horse serum intraspinally. May 19. Repeated 2 hour nasal plug. May 23. Left facial and right arm paralysis. May 24. Prostrate. May 26. Etherized. The medulla and spinal cord showed typical lesions of poliomyelitis.

(b) *Test*.—*Macacus rhesus*. May 14. 2 cc. of normal horse serum injected intraspinally. May 15. 2 hour cotton plug carrying the virus in naris; 10 cc. of pooled monkey poliomyelitic immune serum intravenously. May 16. 2 cc. of normal horse serum intraspinally. May 17. 2 hour plug with virus in naris; 10 cc. of pooled monkey immune serum intravenously. May 18. 2 cc. of normal horse serum intraspinally. May 19. 2 hour plug with virus in naris; 10 cc. of pooled monkey immune serum intravenously. This animal developed no symptoms whatever during several months observation.

Experiment 8. (a) Control.—*Macacus rhesus*. June 4. 2 cc. of normal horse serum intraspinally. June 5. 2 hour cotton plug carrying virus in naris. June 6. 2 cc. of normal horse serum intraspinally. June 7. 2 hour virus-containing nasal plug. June 8. 2 cc. of normal horse serum intraspinally. June 9. 2 hour virus-carrying nasal plug. June 13. Animal weak; no definite paralysis. June 14. Dead. The medulla and spinal cord showed lesions of poliomyelitis.

(b) *Tests*.—Two *Macacus rhesus* monkeys. Procedure identical in both. June 4. 2 cc. of normal horse serum intraspinally. June 5. 2 hour virus-carrying plug in naris. June 6. 10 cc. of pooled monkey poliomyelitic serum intravenously and 2 cc. of normal horse serum intraspinally. June 7. 2 hour virus-carrying nasal plug. June 8. 10 cc. of pooled immune serum intravenously and 2 cc. of normal horse serum intraspinally. June 9. 2 hour nasal plug with virus. June 10. 10 cc. of pooled immune serum intravenously. No symptoms of poliomyelitis developed during the period of observation which extended over several months.

The results of this series of experiments are clear and definite, and show conclusively that even under highly favorable conditions of susceptibility the infection of monkeys with the virus of poliomyelitis applied to the nasal mucosa can be prevented by passive immuniza-

tion of the body by way of the general blood. By this means, therefore, the effective passage of the virus from the nasal mucosa to the central nervous organs can be blocked.

The precise point at which the blocking takes place is in doubt. Two or three possibilities exist: First, after passage of the virus into the blood itself *en route* to the brain and spinal cord. This possibility is small, inasmuch as all the available evidence is against the virus of poliomyelitis reaching the nervous organs from the general blood (Flexner and Amoss¹⁰). Next, in the nasal mucosa itself, as the blood carrying the immune serum circulates through. There is no way of readily affirming or excluding this idea. It seems improbable, however, that the virus in the interstices of the tissue and especially in the olfactory nerves themselves would have been brought under sufficient influence of the immune serum to have been prevented from multiplying and inducing infection. Third, in the central nervous system itself. In our opinion, this last is the more probable site. All the conditions of the experiments are favorable to the passage of a certain amount of the immune serum into the subarachnoid space. Under the influence of the chemical irritant, both the choroid plexus and the meningeal vessels become more pervious to protein substances and hence to the immune bodies (Flexner and Amoss¹²). Once the immune bodies reach the subarachnoid space and mingle with the cerebrospinal fluid, infection with the virus of poliomyelitis injected into the blood or meninges themselves is prevented.

There is little doubt that the quantity of immune serum employed in some of the experiments was excessive. Experiment 6 shows that a single intravenous injection suffices to block the development of the virus. But in the experiments in which repeated nasal tampons were employed and in which several injections of normal horse serum were given, it was deemed advisable to maintain the concentration of the immune serum in the general blood. After all, the answer sought by the experiments was whether under conditions of severe inoculation and a highly favorable degree of susceptibility of the animal tested, blocking of the infection could be secured surely by way of the passively immunized general blood.

¹² Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1917, xxv, 499; 1918, xxviii, 11.

SUMMARY.

1. The experiments given in this paper, notwithstanding their seeming diversity, relate to the conditions underlying the states of susceptibility and refractoriness to infection with the virus of poliomyelitis applied to the nasal mucosa.

2. Certain monkeys are highly refractory to inoculation *via* the nares with the virus of poliomyelitis, apparently in virtue of a power possessed by the nasal mucous membrane to destroy or otherwise render ineffective the virus applied to it.

3. This property of the nasal mucosa appears to be distinct from any specific protective substance active upon the virus which may occur in the blood.

4. An effective nasal mucous membrane prevents the passage of the energetically applied virus to the brain and spinal cord.

5. The virus of poliomyelitis energetically applied to the nasal mucosa will survive for an undetermined period of time upon an ineffective, but for a relatively brief period of time upon an effective membrane.

6. The protective power possessed by the nasal mucosa is not in itself adequate to prevent infection with the virus introduced upon it, since slight injury to such independent structures as the meningeal-choroid plexus complex favors the passage of the virus from the nose to the central nervous organs.

7. The normal nasal mucosa is, therefore, an invaluable defense against infection with the virus of poliomyelitis; and the number of healthy and chronic carriers of the virus is probably determined and kept down through the protective activities of this membrane.

8. Antiseptic chemicals applied to the nasal mucosa upon which the virus has been deposited exhibit no great protective action and are of doubtful value. Indeed, it is not impossible that to the extent to which they may affect unfavorably the destructive properties of the nasal mucosa, they may be even objectionable.

9. Infection with the virus of poliomyelitis applied to the nasal mucosa under conditions favorable to the extension to the central nervous organs and multiplication there may be blocked or prevented by the injection of poliomyelitic immune serum into the blood.

While the exact manner and site of attack of the immune serum upon the virus is somewhat conjectural, when all the available data are considered it seems probable that the meeting place of the virus and immune serum is in the subarachnoid space.

ETIOLOGY OF YELLOW FEVER.

X. COMPARATIVE IMMUNOLOGICAL STUDIES ON *LEPTOSPIRA ICTEROIDES* AND *LEPTOSPIRA ICTEROHÆMORRHAGIÆ*.

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In a previous paper¹ it was reported that serum from convalescent yellow fever patients has a more or less marked antagonistic effect upon *Leptospira icteroides* derived from certain cases of yellow fever in Guayaquil, as manifested by a positive Pfeiffer phenomenon in the peritoneal cavity of the guinea pig. In a few instances the serum protected the guinea pig from a fatal infection with the organism. A similar result was obtained with the serum of guinea pigs which had recovered from a non-fatal experimental infection with the leptospira.

In the present paper the question of immunity will be more fully discussed, particularly with regard to agglutination, lysis, complement fixation, Pfeiffer's reaction, etc., with immune sera prepared in rabbits and horses by repeated inoculations of *Leptospira icteroides*. Experiments have also been carried out to determine the relation between this organism and *Leptospira icterohæmorrhagiæ* of infectious jaundice by means of cross-immunity reactions *in vitro* and *in vivo*. In the *in vivo* experiments not only passive, but also active immunity has been taken into consideration. A part of this study has already been published in a paper dealing with the leptospira isolated from wild rats and mice in Guayaquil² and will not be repeated here.

¹ Noguchi, H., *J. Exp. Med.*, 1919, xxx, 9.

² Noguchi, H., *J. Exp. Med.*, 1919, xxx, 95.

Production of Immune Sera.

Monovalent immune sera for each of four strains of *Leptospira icteroides* were prepared in rabbits by injecting the animals intravenously with 2 to 4 cc. of rich live culture, on rabbit serum medium, several successive times at intervals of 7 to 14 days. The animals showed a definite febrile reaction on the 4th or 5th day after the first injection but no other symptoms. Subsequent inoculations produced no perceptible reaction in most of the animals, although some died suddenly, probably owing to the development of anaphylaxis as the number of inoculations increased.

Polyvalent immune serum was produced in a horse by injecting intravenously (jugular vein) gradually increasing amounts of rich live cultures (horse serum medium) of five strains (Nos. 1, 3, 4, 5, and 6) of *Leptospira icteroides*. 20 cc. of the mixture of cultures were given as the initial injection, and subsequent injections were increased up to 200 cc. This dose was maintained for most of the time during immunization. The first inoculation caused a rise in temperature to 40°C. on the 3rd and 4th days, with considerable swelling along the inoculated side of the neck. The animal lost its appetite during the period but regained its normal condition within 5 days. There was no jaundice at any time. Subsequent inoculations caused no perceptible reaction. During a period of 65 days the horse received 2,495 cc. of mixed live cultures in fifteen injections. The following protocol gives the schedule of immunization.

Horse 2.—Feb. 6, 1919, 20 cc.; Feb. 11, 40 cc.; Feb. 15, 60 cc.; Feb. 19, 125 cc.; Feb. 24, 150 cc.; Mar. 1, 200 cc.; Mar. 6, 200 cc.; Mar. 11, 200 cc.; Mar. 15, 200 cc.; Mar. 19, 200 cc.; Mar. 24, 200 cc.; Mar. 28, 200 cc.; Apr. 2, 200 cc.; Apr. 7, 200 cc.; Apr. 12, 300 cc. First bleeding on Apr. 19, 1919.

Effects of Immune Sera upon Leptospira icteroides and Leptospira icterohæmorrhagiæ.

Monovalent immune sera were prepared in rabbits, as described, and experiments conducted to demonstrate the effects of such sera upon homologous and heterologous strains of *Leptospira icteroides* on the one hand and of *Leptospira icterohæmorrhagiæ* on the other.

The experiments were designed to throw more light on the relation that may exist, not only among different strains of these organisms, but also between *Leptospira icteroides* and *Leptospira icterohæmorrhagica* as distinct varieties. The effects of an immune serum are manifold, but we confined our observations to the phenomena of agglutination, immobilization, and disintegration of the organism when mixed with the immune serum *in vitro* and that of the reaction of Pfeiffer following the simultaneous inoculation of the organism and an immune serum into the peritoneal cavity of guinea pigs. Complement fixation tests were also made.

Agglutination.—The technique employed for the agglutination tests was as follows:

Rich live cultures, grown at 26°C. for 2 to 3 weeks on rabbit serum medium (one part of serum plus three parts of 0.9 per cent sodium chloride solution), of each strain were selected. 1 cc. of each of a number of cultures was put into a series of small sterile test-tubes, and 0.2 cc. of the fresh immune serum to be tested was added to each tube. Controls with normal rabbit serum accompanied each series of experiments. The culture and immune serum were carefully mixed by gentle shaking and the tubes incubated at 37°C. (water bath) for 2 hours. Examinations of the contents were then made by means of the dark-field microscope.

With pronounced agglutination minute particles could be macroscopically detected. Another examination was made on the tubes after they had been left at room temperature for 96 hours, but the results were identical with those recorded after 3 hours, with the possible exception of a more granular appearance of some of the agglutinated cultures and in extreme instances a thin but definite grayish sediment at the bottom of the test-tubes.

In the present series of experiments there were available three monovalent antisera for *Leptospira icteroides*, comprising Strains 1, 5, and 6, and six for *Leptospira icterohæmorrhagica*, comprising the Japanese, British, American No. 1, Group 8, Group 11, and Group 30 strains. Against each of these nine monovalent immune sera were tested cultures of five different strains of *Leptospira icteroides*, Nos. 1, 3, 4, 5, and 6, and seven strains of *Leptospira icterohæmorrhagica*, Japanese, British, French, American No. 1, Group 8, Group 11, and Group 30.

A strong immune serum acting upon the homologous strain of *Leptospira icteroides* agglutinated the organisms quickly into rather large masses, in which they appeared tightly held together. Most of the organisms became immobile, gradually lost their elementary windings, and were soon transformed into stiff, irregularly granular filaments. If the serum was not strong enough to produce these changes, the agglutinated masses were for the most part degenerated, but with several apparently intact immobilized organisms at the periphery. In other instances the agglutinated masses contained a certain number of individuals which were still active, while in still others the organisms in agglutinated masses showed quivering motility in some part of their body. On the whole, agglutination is the first and more constant reaction observed and disintegration the secondary and less constant (Tables I and II).

Pfeiffer's Phenomenon.—To complete the observations, Pfeiffer tests were also performed with the immune sera and the various strains of *icteroides* and *icterohæmorrhagiæ*. The technique employed was that generally followed. 1 cc. of a given immune serum was mixed in a Petri dish with 1 cc. of a rich live culture and immediately injected into the peritoneal cavity of a normal guinea pig. The peritoneal fluid was withdrawn with a capillary pipette after 30 minutes and 2 hours and examined under the dark-field microscope (Tables I and II).

The reaction of Pfeiffer with the immune sera and homologous strains of *Leptospira icteroides* is prompt and complete. The organisms seem first to be agglutinated into large masses and then to be quickly disintegrated. The phenomenon may be complete within 15 minutes, so that no trace of the organisms can be seen in the peritoneal fluid. The same is true of *Leptospira icterohæmorrhagiæ* and the homologous immune sera. A decided increase of actively motile organisms was noticed in the guinea pig peritoneal cavity when a normal rabbit serum instead of a specific immune serum was used.

As shown in Table I, the five different strains of *Leptospira icteroides* reacted to each of the three monovalent immune sera. The intensity of agglutination and disintegration varied somewhat according to whether the strains were homologous or heterologous. Without

Effects of Monovalent Anti-icteroides Sera upon Leptospira icteroides and Leptospira icterohæmorrhagæ.

* The varying degrees of agglutination and disintegration are recorded by plus signs vertically placed; their absence by a minus sign.
+ indicates positive; ± doubtful; — negative.

TABLE II.
Effects of Monovalent Anti-icterohemorrhagic Sera upon *Leptospira icterohemorrhagica* and *Leptospira icteroides*.

Serum.	Strain of <i>Leptospira icterohemorrhagica</i> .												Strain of <i>Leptospira icteroides</i> .																							
	Japanese.			British.			French.			American No. 1.			Group 8.			Group 11.			Group 30.			No. 1.			No. 3.			No. 4.			No. 5.			No. 6.		
	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.			
Immune serum for <i>Leptospira icterohemorrhagica</i> , Japanese strain.	++	++	+	++	++	+	++	++	+	++	++	+	++	++	+	++	++	+	++	++	+	++	++	+	++	++	+	++	++	+	++	++	+			
Immune serum for <i>Leptospira icterohemorrhagica</i> , British strain.	++	++	+	++	++	+	++	++	+	++	++	+	++	++	+	++	++	+	++	++	+	++	++	+	++	++	+	++	++	+	++	++	+			
Immune serum for <i>Leptospira icterohemorrhagica</i> , American Strain 1.	++	++	+	++	++	+	++	++	+	++	++	+	++	++	+	++	++	+	++	++	+	++	++	+	++	++	+	++	++	+	++	++	+			
Immune serum for <i>Leptospira icterohemorrhagica</i> , Group 8 strain.	++	++	+	++	++	+	++	++	+	++	++	+	++	++	+	++	++	+	++	++	+	++	++	+	++	++	+	++	++	+	++	++	+			

Immune serum for <i>Leptospira icterohaemorrhagiae</i> , Group 11 strain.	—	—	—
	—	—	—
	—	—	—
	—	—	—
	—	—	—
	+	+	—
	—	—	—
	—	—	—
	—	—	—
	+	—	—
Immune serum for <i>Leptospira icterohaemorrhagiae</i> , Group 30 strain.	+	—	—
	+	—	—
	+	—	—
	+	—	—
	+	—	—
	+	—	—
	+	—	—
	+	—	—
	+	—	—
	+	—	—
Normal rabbit serum (control).	+	—	—
	+	—	—
	+	—	—
	+	—	—
	+	—	—
	+	—	—
	+	—	—
	+	—	—
	+	—	—
	+	—	—

exception the strongest reaction was obtained with the homologous and a less pronounced one with heterologous strains. Disintegration of the organisms was usually complete in the homologous but seldom so in heterologous combinations. Normal rabbit serum exerted neither an agglutinating nor a disintegrating influence upon any of the strains studied. On the contrary, the addition of normal rabbit serum to control tubes kept the organisms active for many days.

That these anti-*icteroides* sera did not agglutinate the various strains of *Leptospira icterohæmorrhagiæ* to any marked degree is also shown in this table. There were a few instances in which slight agglutination was observed, but none so marked as that which occurred with the strains of *icteroides*. In contrast to the results obtained with the *icteroides* strains, in no instance was there any disintegration of the *icterohæmorrhagiæ* organisms by an anti-*icteroides* serum. The Pfeiffer phenomenon was invariably positive with the anti-*icteroides* serum and the *icteroides* strains, but almost always negative when the anti-*icteroides* serum was tested with the *icterohæmorrhagiæ* strains. In two instances there was a suggestive reaction.

Table II presents the results obtained with six different monovalent anti-*icterohæmorrhagiæ* sera. The marked difference that exists between the *icterohæmorrhagiæ* strains and the *icteroides* strains is shown. Aside from slight variations, the *icterohæmorrhagiæ* strains reacted with the *icterohæmorrhagiæ* antisera quite generally and strongly and present a marked contrast to the *icteroides* strains, which reacted occasionally and never very strongly. The Pfeiffer phenomenon was positive in all combinations of the anti-*icterohæmorrhagiæ* serum with the *icterohæmorrhagiæ* group, but only occasionally and slightly with the *icteroides* group. The occurrence of a fairly marked agglutination in certain instances, such as, for example, anti-Japanese serum *versus* No. 1 *icteroides* strain or anti-group No. 11 serum *versus* No. 3 *icteroides* strain, is of considerable interest because of the occurrence of similar weak reactions among the combinations of anti-*icterohæmorrhagiæ* sera and certain heterologous *icterohæmorrhagiæ* strains. The only reliable differentiation in these instances would seem to be that of the Pfeiffer test. Beyond these few irregularities the behavior of the immune sera towards *icterohæmorrhagiæ* and *icteroides* seems to warrant the conclusion that the strains of

Leptospira icteroides and *Leptospira icterohæmorrhagiæ* form closely related but distinct groups.

Complement Fixation.—The technique used in the complement fixation tests was as follows:

Rich cultures of various strains of *Leptospira icteroides* and *Leptospira icterohæmorrhagiæ* were grown on rabbit serum media, killed by placing the culture tubes in a water bath at 60°C. for 10 minutes, and then used as antigens in the complement fixation tests. Graduated quantities (0.1, 0.05, 0.02, 0.01, 0.005 cc.) of each of the monovalent sera were mixed with a uniform quantity of the antigen (a quantity which had been found to be non-anticomplementary in several doses when tested with 0.1 cc. of complement), and to each tube was added 0.1 cc. of fresh guinea pig serum as complement. The mixtures were brought up to a total volume of 1.5 cc. for each tube by the addition of 0.9 per cent saline solution. After incubation at 37°C. for 1 hour, 0.1 cc. of a 20 per cent suspension of washed sheep corpuscles and 0.1 cc. of anti-sheepamboceptor (rabbit serum), representing three hemolytic units, were added to each tube, the contents thoroughly mixed, and once more incubated at 37°C. for 30 minutes. The results were read after standing for 1 hour. The actual quantity of each antigen used was 0.1 cc. of the killed culture, which exhibited only a slight anticomplementary property when used alone in quantities of from 0.4 to 0.6 cc.

In the majority of instances the reaction was maximum with 0.1 cc. of immune serum, and all tests with 0.005 cc. gave a negative result. The readings of the reaction obtained with 0.1 cc. of the antigens and 0.1 cc. of the immune sera are recorded in Table III.

Complete fixation took place when the immune sera were mixed with the homologous strains, both in the case of *Leptospira icteroides* and in that of *Leptospira icterohæmorrhagiæ*. Occasionally, especially among the *icteroides* strains, the fixation was as strongly positive with one or the other of the heterologous strains as with the homologous. There were a number of instances also in which a more or less definite fixation occurred when the anti-*icteroides* sera were mixed with the *icterohæmorrhagiæ* strains or the anti-*icterohæmorrhagiæ* sera with the *icteroides* strains, some reactions being as strong as \pm . Generally speaking, however, there was only a limited degree of cross-reaction between *icteroides* and *icterohæmorrhagiæ*.

Variations in the fixation reaction were rather marked among the different strains of *icterohæmorrhagiæ*, according to the combinations of the immune sera and heterologous strains, some of which showed only a feeble fixation (+) with certain sera.

TABLE III.

Complement Fixation Tests.

Serum.	Strain of <i>Leptospira icteroides</i> .					Strain of <i>Leptospira icterohæmorrhagæ</i> .						
	No. 1.	No. 3.	No. 4.	No. 5.	No. 6.	Japanese.	British.	French.	American No. 1.	American No. 2.	American No. 3.	Group 8.
Immune serum for <i>Leptospira icteroides</i> , Strain 1.	+	+	+	+	+	+	-	-	-	-	-	-
	+	+	+	+	+							
	+			+								
	+			+								
Immune serum for <i>Leptospira icteroides</i> , Strain 5.	+	+	+	+	+	-	-	-	+	+	-	+
	+	+	+	+	+							
	+	+		+								
	+			+								
Immune serum for <i>Leptospira icteroides</i> , Strain 6.	+	+	+	+	+	-	-	+	-	-	+	+
	+	+	+	+	+							
	+	+	+		+							
	+	+	+		+							
Immune serum for <i>Leptospira icterohæmorrhagæ</i> , Japanese strain.	+	±	+	-	-	+	+	+	+	+	+	+
						+	+	+	+	+	+	+
						+	+	+	+	+	+	+
						+	+	+	+	+	+	+
Immune serum for <i>Leptospira icterohæmorrhagæ</i> , British strain.	-	-	-	-	-	+	+	+	+	+	+	+
						+	+	+	+	+	+	+
						+	+	+	+	+	+	+
						+	+	+	+	+	+	+
Immune serum for <i>Leptospira icterohæmorrhagæ</i> , American Strain 1.	-	+	+	-	-	+	+	+	+	+	+	+
						+	+	+	+	+	+	+
						+	+	+	+	+	+	+
						+	+	+	+	+	+	+
Immune serum for <i>Leptospira icterohæmorrhagæ</i> , Group 8 strain.	-	-	-	-	-	+	+	+	+	+	+	+
						+	+	+	+	+	+	+
						+	+	+	+	+	+	+
						+	+	+	+	+	+	+

TABLE IV.
Protective Properties of Monovalent Immune Rabbit Sera against Leptospira icteroides and Leptospira icterohemorrhagiae.

Serum.	Strain of <i>Leptospira icteroides</i> .		Strain of <i>Leptospira icterohemorrhagiae</i> .			
	No. 5.	No. 6.	Japanese.	French.	Guayaquil No. 8.	Guayaquil No. 30.
Normal rabbit serum (control).	One guinea pig died in 7 and the other in 6 days.	Died in 6 days.	Died in 9 days. Typical symptoms and lesions.	Died in 10 days. Typical symptoms and lesions.	Two guinea pigs died in 7 days. Typical lesions.	Two guinea pigs died in 8 days. Typical symptoms and lesions.
Immune Serum 945. Homologous with <i>Leptospira icteroides</i> , Strain 5.	Survived. No symptoms.	Survived. No symptoms.	Survived; severe infection with marked lesions.	Survived; severe infection.	Died in 9 days. Typical lesions.	Died in 11 days. Typical symptoms and lesions.
Immune Serum 942. Homologous with <i>Leptospira icteroides</i> , Strain 6.	Survived. No symptoms.	Survived. No symptoms.	Died in 9 days. Typical symptoms and lesions.	Survived; severe infection. Marked lesions.	Died in 8 days. Typical lesions.	Died in 10 days. Typical symptoms and lesions.
Immune Serum 911. Homologous with <i>Leptospira icterohemorrhagiae</i> , Japanese strain.	Died in 10 days. Typical symptoms and lesions.	Died in 10 days. Typical symptoms and lesions.	Survived. Slight lesions.	Survived. Examination showed a few lung lesions.	Survived. No symptoms.	Survived. No symptoms.

Immune Serum 947. Homologous with <i>Leptospira ictero- hemorrhagica</i> , British strain.	Died in 9 days. Typical symp- toms and le- sions.	Died in 12 days. Typical symp- toms and le- sions.	Survived. Slight lesions.	Survived. lesions.	No symptoms.	Survived. No infection.
Immune Serum 952. Homologous with <i>Leptospira ictero- hemorrhagica</i> , American Strain 1.	Died in 11 days. Typical symp- toms and le- sions.	Survived; mild infection.	Survived; mild infection.	Survived. lesions.	Survived; mild infection.	Survived.
Immune Serum 904. Homologous with <i>Leptospira ictero- hemorrhagica</i> , Guayaquil Strain 30.	Survived; severe infection.	Died of intercur- rent disease.	Survived. Slight lesions.	Survived. lesions.	Survived. No symptoms.	"

severe infection in guinea pigs when mixed with normal rabbit serum or with one or another of the antisera produced with different strains of *Leptospira icterohæmorrhagiæ*. It is noteworthy that the period of incubation when anti-*icterohæmorrhagiæ* serum and *Leptospira icteroides* are combined is somewhat prolonged. For example, with Strain 5 of *icteroides* the control animals with normal rabbit serum died in 6 or 7 days, while the guinea pigs receiving the anti-*icterohæmorrhagiæ* sera died between 9 and 11 days after the inoculation. This is also true of Strain 6, with which death occurred in 6 days in the control animal and in 10 and 12 days in the animals injected with the anti-*icterohæmorrhagiæ* sera. The symptoms and lesions were typical in all instances in which an infection ensued.

The results obtained by reversing the combinations, that is by mixing different strains of *Leptospira icterohæmorrhagiæ* with anti-*icteroides* immune sera, show also an unmistakable specificity of the protection afforded by these immune sera. A clear-cut specific protection is shown in the experiments with the Japanese and Guayaquil strains of *icterohæmorrhagiæ*; these three strains were effectively neutralized by their homologous immune sera, but not by any anti-*icteroides* immune sera, although the sera seemed to delay death in some instances. The French strain of *icterohæmorrhagiæ* was least virulent and did not cause fatal infection in guinea pigs when any one of the immune sera was simultaneously inoculated. The examination of the surviving guinea pigs for the lesions, particularly those in the lungs, after 24 days showed that the guinea pigs which received the anti-*icteroides* sera had numerous old hemorrhagic foci in the lungs, while none or only a few foci were found in those which were inoculated with the anti-*icterohæmorrhagiæ* sera.

Polyvalent Immune Horse Sera.—A horse was immunized with a mixture of cultures of four strains of *Leptospira icteroides* for a period of 65 days, during which 2,495 cc. of the cultures were injected intravenously as described in the protocol above. At the same time another horse, which had once been immunized for a period of several months in 1918 with various strains of *Leptospira icterohæmorrhagiæ*, was injected again, with *icterohæmorrhagiæ* cultures, comprising nine strains: Japanese (one strain), American (three strains), French (one strain), British (one strain), and Guayaquil (three strains).

The serum from each horse was then tested for its protective property in guinea pigs against some of the representative strains of *Leptospira icteroides* and *Leptospira icterohæmorrhagiæ*.

The procedure consisted in injecting into the peritoneal cavity of guinea pigs a mixture of a given quantity of culture (or liver emulsion) of a strain with 1 cc. of immune serum, either full strength or diluted, as indicated in Tables V and VI.

For each dose of the serum two guinea pigs were used in order to determine its titer as closely as possible. Only one guinea pig, however, was used in determining the protective titers of the anti-*icteroides* serum against the *icterohæmorrhagiæ* strains or *vice versa*. The amounts of culture (or liver emulsion) used in testing the corresponding antiserum were such as to approximate 500 minimum lethal doses, while for a cross-titration usually about 50 minimum lethal doses, or even 10 minimum lethal doses were chosen. The reason for reducing the number of minimum lethal doses in cross-protection experiments was that in several preliminary tests a larger quantity of culture was found not to be influenced to any great extent by a heterologous antiserum.

The anti-*icteroides* serum protected guinea pigs against approximately 500 minimum lethal doses of *Leptospira icteroides* in doses of 0.001 (for Strain 5) and 0.0001 cc. (for Strain 6). In other words, 1 cc. of this serum neutralizes about 500,000 to 5,000,000 minimum lethal doses of *Leptospira icteroides*, according to the degree of virulence of the culture. On the other hand, at least 1 cc. of the same serum was required to protect guinea pigs against 10 to 50 minimum lethal doses of the Japanese and French strains of *Leptospira icterohæmorrhagiæ*. 0.1 cc. of the serum averted a fatal outcome but failed to prevent wholly the infection. The difference in the protective efficacy of the anti-*icteroides* serum against *icteroides* and *icterohæmorrhagiæ* is striking.

The anti-*icterohæmorrhagiæ* serum exhibited also a marked specific protective property for the *Leptospira icterohæmorrhagiæ* strains, neutralizing at least 5,000,000 (for the French) to 500,000 (for the Japanese) minimum lethal doses per 1 cc. Its effect upon the *icteroides* strains was in each case feeble but distinct, since it protected guinea pigs completely against 50 minimum lethal doses per 1 cc.

TABLE V.

Protective Properties of Polyvalent Anti-icteroides Serum against Leptospira icteroides and Leptospira icterohamorrhagiae.

Anti-icteroides serum.	Strain of <i>Leptospira icteroides</i> .		Strain of <i>Leptospira icterohamorrhagiae</i> .	
	No. 5. 0.1 cc. of culture (about 500 M. L. D.).	No. 6. 0.1 cc. of culture (about 500 M. L. D.).	Japanese. 0.1 cc. of liver emulsion (about 10 M. L. D.).	French. 0.1 cc. of culture (about 50 M. L. D.).
cc.				
0.000001	Died in 7 days. Typical symptoms and lesions.	Died in 7 days.	Died in 7 days.	Died in 9 days.
0.000001	Died in 9 days. Typical symptoms and lesions.	" " 10 "		
0.00001	Survived.	" " 9 "	Died in 6 days.	Died in 10 days.
0.00001	Died in 9 days.	Survived.		
0.0001	" " 9 "	"	Survived (!).	Died in 8 days.
0.0001	Survived.	"		
0.001	"	"	Died in 14 days.	Died in 13 days.
0.001	"	"		
0.01	"	"	Died in 10 days.	Died in 13 days.
0.01	"	"		
0.1	"	"	Survived. Had fever.	Survived. Had fever.
0.1	"	"		
1.0	"	"	Survived. Had fever.	Survived. Had fever.
1.0	"	"		
Normal horse serum 1.0 cc. (control).	Died in 7 days. Typical symptoms and lesions.	Died in 7 days. Typical symptoms and lesions.	Died in 7 days. Typical symptoms and lesions.	Died in 8 days.

and prevented death, but not infection, when used in a dose of 0.1 cc. It will be seen, therefore, that the respective polyvalent antisera exert a powerful annihilating effect in guinea pigs upon their corresponding type organisms, but there also exists an undeniable, though

feeble, cross-protective reaction, which may be explained by assuming that the two groups of organisms are not altogether alien but are closely related to each other; they may even constitute two subspecies or races.

TABLE VI.

Protective Properties of Polyvalent Anti-icterohæmorrhagiæ Serum against Leptospira icterohæmorrhagiæ and Leptospira icteroides.

Anti-icterohæmorrhagiæ serum.	Strain of <i>Leptospira icterohæmorrhagiæ</i> .		Strain of <i>Leptospira icteroides</i> .	
	Japanese. 0.1 cc. of culture (about 500 M. L. D.).	French. 1 cc. of culture (about 500 M. L. D.).	No. 1. 0.1 cc. of culture (about 50 M. L. D.).	No. 5. 0.01 cc. of culture (about 50 M. L. D.).
cc.				
0.000001	Died in 6 days.	Died in 10 days.	Died in 8 days.	Survived (!).
0.000001	" " 7 "	" " 13 "		
0.00001	" " 6 "	Survived.	Died in 7 days.	Died in 8 days.
0.00001	" " 11 "	Died in 11 days.		
0.0001	Survived.	Survived.	Died in 8 days.	Died in 8 days.
0.0001	Died in 8 days.	"		
0.001	Survived.	"	Died in 7 days.	Died in 9 days.
0.001	"	"		
0.01	"	"	Died in 8 days.	Died in 15 days.
0.01	"	"		
0.1	"	"	Survived; fever.	Survived; mild jaundice.
0.1	"	"		
1.0	"	"	Survived.	Survived.
1.0	"	"		
Normal horse serum 1.0 cc. (control).	Died in 7 days.	Died in 7 days.	Died in 6 days.	Died in 7 days.

Active Immunity.

Guinea pigs vary considerably in their susceptibility to *Leptospira icteroides*. It sometimes happens that a culture which kills guinea pigs of average susceptibility in a dose of about 0.0001 cc. occasionally

fails to produce a fatal infection in a guinea pig in a dose as large as 0.01 or 0.1 or even 1 cc. Such refractory guinea pigs are rarely met with, but the fact that there exist certain unusually resistant individuals is of great interest. It has also been found that there are exceptionally susceptible individuals which respond to infection with an attenuated culture which no longer attacks the average guinea pig. For example, with certain cultures of *Leptospira icteroides* which had been repeatedly subcultured without passage through the guinea pig for many months, only one out of several animals inoculated with the same culture may come down with typical symptoms. In fact, when the first attempt to restore the virulence of the culture by animal passage failed, a second or third attempt with four or five guinea pigs each time was necessary to obtain a single positive result. These facts furnish possible explanations for certain paradoxical results which are sometimes encountered in determining the state of immunity.

In a discussion of active immunity we may distinguish between that which arises from recovery from a genuine infection and that which follows the inoculation of killed organisms. Animals which, after receiving an inoculation of a sublethal dose of a live culture, do not react definitely, may acquire a state of immunity similar to that of vaccinated animals.

The results with the guinea pigs which had recovered from a more or less pronounced infection after the inoculation of a culture or blood derived from a guinea pig dying of the typical infection with *Leptospira icteroides* will first be described. The tests consisted in the Pfeiffer phenomenon and the effect of the inoculation of different cultures in case of a negative Pfeiffer reaction.

Series 1.

Four guinea pigs were actively immunized with Strain 5 of *Leptospira icteroides*.

Guinea Pig Ch 1.—Nov. 27, 1918. Received 1 cc. of citrate blood from a guinea pig which had been infected with Strain 5 of *Leptospira icteroides* and showed the typical symptoms of *icteroides* infection. After the usual course of infection (fever, slight icterus, albuminuria) the animal became well within 2 weeks. Dec. 19. Received 1 cc. of culture of the same strain. No symptoms followed. Jan. 6, 1919. Received another injection of culture of the same strain, without any perceptible effect.

Pfeiffer Reaction.—Jan. 18. A rich culture of the same strain (1 cc.) was injected intraperitoneally. A prompt positive reaction was obtained. Jan. 22. The Pfeiffer reaction was tested with Strain 6, 1 cc. of a rich culture being used. Result positive. On the same day, 4 hours later, another Pfeiffer test was made on this animal with the Japanese strain of *Leptospira icterohæmorrhagiæ*, 1 cc. of culture being used. Result negative. This animal died in 23 days with the typical symptoms of *icterohæmorrhagiæ* infection.

Guinea Pig Ch 2.—The procedure with this animal was similar to that with Guinea Pig Ch 1.

Pfeiffer Reaction.—Jan. 18, 1919. Tested with 1 cc. of culture of the same strain. Result positive. Jan. 22. Pfeiffer test with 1 cc. of Strain 6 culture was positive. The same day, 4 hours later, tested with 1 cc. of French strain of *Leptospira icterohæmorrhagiæ*. Result negative. This animal died in 14 days with the typical symptoms of *icterohæmorrhagiæ* infection.

Guinea Pig Ch 3.—The procedure with this animal was similar to that with Guinea Pig Ch 1.

Pfeiffer Reaction.—Jan. 18, 1919. Test with 1 cc. of the same strain was positive. Jan. 22. Test with 1 cc. of the British strain of *Leptospira icterohæmorrhagiæ* was doubtful. The animal survived.

Guinea Pig Ch 4.—The procedure with this animal was similar to that with Guinea Pig Ch 1.

Pfeiffer Reaction.—Jan. 18, 1919. With 1 cc. of culture of the same strain test was positive. Jan. 22. With 1 cc. of culture of Strain 6 of *Leptospira icteroides* test was positive. The animal survived.

Series 2.

Three guinea pigs, out of a large number inoculated with Strain 6 of *Leptospira icteroides*, which suffered more or less severe infection and eventually recovered, were reinoculated with a culture of the same strain once or twice (as described in the protocols below) afterwards, but showed no characteristic symptoms, except a rise of temperature for a day in one (Guinea Pig C 2). Hence they were completely immune to the same strain and were used to test their resistance to certain other strains, including the Japanese strain of *icterohæmorrhagiæ*.

Guinea Pig C 1.—Dec. 3, 1918. Received 1 cc. of Strain 6 culture of *Leptospira icteroides*. The animal had typical fever and a trace of jaundice, but recovered in about 9 days. Two more injections of 1 cc. of the same culture were given, one on Dec. 19, and another on Jan. 6, 1919.

Pfeiffer Reaction.—Jan. 18. With 1 cc. of a rich culture of the same strain, a prompt and positive reaction. Jan. 22. Inoculated with 1 cc. of a rich culture of Strain 5 of *Leptospira icteroides*. Pfeiffer reaction positive. No symptoms within 1 month.

Guinea Pig C 2.—Dec. 11, 1918. Received 1 cc. of blood from a guinea pig showing typical symptoms (fever and slight jaundice). The animal eventually

recovered. Jan. 6, 1919. 1 cc. of a rich culture of the same strain was given. There was slight fever for a day, but no infection followed.

Pfeiffer Reaction.—Jan. 18. 1 cc. of a culture of the same strain was inoculated intraperitoneally, but no organism could be found in the peritoneal exudate after 30 minutes. Jan. 22. The animal was tested again for the Pfeiffer reaction, with 1 cc. of a rich culture of Strain 1 of *Leptospira icteroides*. There was a complete positive reaction within 30 minutes, and no infection followed the inoculation.

Guinea Pig C 3.—This animal was immunized in the same way as Guinea Pig C 2.

Pfeiffer Reaction.—Jan. 18, 1919. 1 cc. of a rich culture of the same strain was injected intraperitoneally. Reaction prompt and complete. Jan. 22. 1 cc. of a rich culture of the Guayaquil strain, Group 30, of *Leptospira icterohæmorrhagiæ* was inoculated intraperitoneally. When examined after 2 hours the organisms were partially agglutinated, but most of them were actively motile. On the 4th day the temperature rose and remained above normal for 3 days. There was a slight jaundice on the 9th and 10th days, which soon faded. The animal survived. In this instance there existed a mild infection with the *icterohæmorrhagiæ* strain.

Series 3.

Nov. 27, 1918. Three guinea pigs were inoculated with a culture of the Japanese strain of *Leptospira icterohæmorrhagiæ*. A second injection was given on Dec. 19, and a third on Jan. 6, 1919. The animals showed a definite but mild infection after the first inoculation, but recovered. No symptoms followed the second or third injection of the same strain.

Guinea Pig J 1. Pfeiffer Reaction.—Jan. 18, 1919. With a culture of the Japanese strain, test positive. The animal remained well.

Guinea Pig J 2. Pfeiffer Reaction.—Jan. 18, 1919. With a rich culture of Strain 5 of *Leptospira icteroides*, test negative. The animal survived, passing through a moderately severe *icteroides* infection.

Guinea Pig J 3. Pfeiffer Reaction.—Jan. 18, 1919. Pfeiffer reaction with a rich culture of Strain 6 of *Leptospira icteroides* not clear-cut. There was a tendency to formation of agglomerated masses, without immobilization or lysis of the organisms. The animal showed mild but typical symptoms of *icteroides* infection after 13 days, but eventually recovered.

Supplementary Experiment with Anti-icterohæmorrhagiæ Serum.

Jan. 16, 1919. A number of guinea pigs were inoculated with mixtures of a polyvalent anti-*icterohæmorrhagiæ* horse serum prepared by Inada and Ido and different strains of *Leptospira icterohæmorrhagiæ* and *Leptospira icteroides*, with a view to determining the protective property of this serum against the *icterohæmorrhagiæ* as well as the *icteroides* strains. 1 and 0.1 cc. of the serum were used, mixed with 0.5 cc. of a culture of each strain, and inoculated at once into the peritoneal cavity

of guinea pigs. The protective titer of the serum had previously been tested against the Japanese strain of *Leptospira icterohæmorrhagiæ* and the serum had been found to neutralize 1 cc. of the culture in a dose of 0.001 cc. The result of the present experiment showed that the guinea pigs which received the immune serum and cultures of the Japanese, French, American, and Guayaquil strains of *icterohæmorrhagiæ* survived, while the control animals without the serum died in 6, 9, 8, and 7 days respectively. There were no symptoms observed in the surviving animals at any time; they were completely protected by the serum in the doses given (1 and 0.1 cc.). All the guinea pigs inoculated with a culture of the British strain of *icterohæmorrhagiæ* with or without (one control) the addition of the immune serum survived; that is, the culture employed was apparently avirulent.

The result obtained with the strains of *Leptospira icteroides* was somewhat surprising. The strains used in this group were Nos. 5 and 6, which killed the control animals in 5 and 10 days respectively. Both cultures, however, produced only a temporary febrile reaction when injected together with 1 cc. of the serum and a moderately severe but non-fatal infection with 0.1 cc.

All the guinea pigs surviving in this series of experiments were subjected on Feb. 10, 25 days after the first inoculation, to immunity tests with cultures of various strains. The guinea pig which had received on Jan. 16 the mixture of the Japanese strain and 1 cc. of anti-*icterohæmorrhagiæ* serum escaped infection and was inoculated with 0.5 cc. of a culture of Strain 5 of *Leptospira icteroides* on Feb. 10. It died with typical symptoms of *icteroides* infection in 7 days. The guinea pig which escaped infection with the mixture of the French strain and 1 cc. of anti-*icterohæmorrhagiæ* serum on Jan. 16 and was injected with 0.5 cc. of Strain 5 of *icteroides* on Feb. 10 died in 5 days with typical symptoms of *icteroides* infection. The guinea pig which escaped infection with the mixture of American Strain 1 and 1 cc. of anti-*icterohæmorrhagiæ* serum on the first injection and was inoculated with 0.5 cc. of Strain 5 of *icteroides* on Feb. 10 finally recovered after a moderately severe *icteroides* infection.

As already mentioned, the guinea pigs which were inoculated on Jan. 16 with 1 and 0.1 cc. of anti-*icterohæmorrhagiæ* serum, together with the cultures of Strains 5 and 6 of *Leptospira icteroides*, had a temporary febrile reaction or a moderately severe infection. These animals were injected on Feb. 10, 25 days later, with a culture of the Japanese strain. All except one, which showed only slight lesions in the lungs, died with the typical symptoms of *icterohæmorrhagiæ* infection in 8 to 9 days. This would seem to indicate that these animals were protected by the anti-*icterohæmorrhagiæ* serum from the fatal outcome of the *icteroides* inoculations, but they were not rendered immune against the Japanese strain of *icterohæmorrhagiæ* when tested 25 days afterwards. Also the anti-*icterohæmorrhagiæ* serum injected on the first occasion had no perceptible protective action against this strain after a period of 25 days.

SUMMARY AND CONCLUSIONS.

It has been previously reported³ that a filterable microorganism belonging to the genus *Leptospira* has been recovered from the blood or organs of human beings suffering from the disease known as yellow fever in Guayaquil, and that the organism, which has been termed *Leptospira icteroides*, induces in certain experimental animals the characteristic symptoms and lesions observed in the patients from whom it was isolated. It has also been previously shown¹ that the serum from patients recovering from an attack of yellow fever in Guayaquil had the power to agglutinate and dissolve the organism when introduced into the peritoneal cavity of a normal guinea pig (Pfeiffer phenomenon). Moreover, the guinea pigs which had once been inoculated with the blood of yellow fever patients without succumbing to the infection, notwithstanding the fact that they had shown a definite febrile reaction after 4 to 5 days, were found to be refractory to a subsequent inoculation of a culture of *Leptospira icteroides*.⁴ All these observations pointed to the possible relation of this organism to the disease known as yellow fever in Guayaquil. The demonstration of the filterability of the organism⁵ and the transmission of the infection with the same organism by *Stegomyia calopus*⁶ have further strengthened the probable etiological significance of the organism in yellow fever.

It was by no means a simple problem to determine the relation existing between *Leptospira icteroides* and *Leptospira icterohæmorrhagiæ*. An experiment reported in a previous paper seemed to justify the view that the two leptospires⁷ are closely related but not identical, yet it was necessary to exhaust various other modes of differentiation before the distinction between them was firmly established. The present paper continues this phase of the inquiry in further detail.

There have been taken up here the phenomena of agglutination, the reaction of Pfeiffer, complement fixation, the protective properties of various monovalent and polyvalent immune sera, and active

³ Noguchi, H., *J. Exp. Med.*, 1919, xxix, 547, 565, 585; xxx, 87.

⁴ Noguchi, H., *J. Exp. Med.*, 1919, xxx, 1.

⁵ Noguchi, H., *J. Exp. Med.*, 1919, xxx, 13.

⁶ Noguchi, H., *J. Exp. Med.*, 1919, xxx, 401.

⁷ Noguchi, H., *J. Exp. Med.*, 1919, xxx, 95.

immunity. As the result of experiments in connection with these immunity phenomena the following data are presented.

Monovalent immune sera prepared by several successive injections in an animal naturally refractory to *Leptospira icteroides* possess the power to agglutinate *in vitro* not only the homologous strains, but also all other strains of *icteroides* tested. On the other hand, a slight effect, or none at all, has been observed when these immune sera have been mixed *in vitro* with various strains of *Leptospira icterohæmorrhagiæ*. A similar relation exists between the monovalent anti-*icterohæmorrhagiæ* sera and the various strains of *Leptospira icteroides*; that is, there is a slight agglutinating effect in some instances upon the *icteroides* strains, but it is never so strong as that occurring in tests against the *icterohæmorrhagiæ* strains. The Pfeiffer reaction gave a sharper differentiation between the two groups, for in most instances the phenomenon was specific for the group. There were occasional doubtful reactions, but not enough to warrant a confusion of the two groups.

Polyvalent immune sera, one specific for *icteroides*, and the other for *icterohæmorrhagiæ*, showed a high titer of neutralizing power for the cultures of the homologous groups. It was found, however, that the action of the sera is by no means absolutely specific, because the injection of a sufficient amount of the anti-*icteroides* serum apparently prevented a fatal outcome in a guinea pig inoculated with multiple minimum lethal doses of a culture of *Leptospira icterohæmorrhagiæ*, and *vice versa*. The specificity of the serum was demonstrated only when it was used in smaller quantities.

More or less specificity was shown by the complement fixation reaction, but it was not absolute. Weak fixation occurred when the anti-*icteroides* serum was mixed with one or the other of the *icterohæmorrhagiæ* strains and *vice versa*, and strong fixation occurred only when the antiserum was mixed with one of the *icteroides* strains. The question naturally arises whether or not this apparent specificity is due to the homology of the serum and not altogether to a difference in genus of the strains. In other words, it is justifiable to question whether all these variations in the degree of intensity of the reaction are not due to strain variations of the same genus. This question is not finally settled by the present investigation, in which only four

icteroides and nine *icterohæmorrhagiæ* strains have been carefully studied. Nevertheless, on the basis of the findings with these thirteen strains, it seems probable that *Leptospira icteroides* and *Leptospira icterohæmorrhagiæ* are closely allied but are nevertheless distinct in their immunological reactions. Perhaps the difference between the two may amount to that between subspecies or races. It has been pointed out earlier that the pathogenicity of the two is also distinct, inasmuch as *icteroides* produces chiefly icterus and nephritis and *icterohæmorrhagiæ* hemorrhage and nephritis, the icterus being less and the hemorrhage more prominent in the evolution of the latter infection.

In the study of active immunity—exclusive of vaccination—difficulty has been experienced in the evaluation of the results, owing to the existence of natural resistance to infection among guinea pigs. A guinea pig may recover from the inoculation of *Leptospira icteroides* and then resist a subsequent inoculation with a virulent strain of *Leptospira icterohæmorrhagiæ*, a condition simulating that brought about by the identity of the two organisms. However, the refractoriness of such an animal to *icterohæmorrhagiæ* may be due to its natural immunity to it. In the present study, therefore, only those guinea pigs were selected which had reacted typically—though in mild degree—to the *icteroides* infection, in order to determine whether they were subsequently immune to the inoculation of *icterohæmorrhagiæ*. Indeed, by this mode of experimentation it was found that the guinea pigs which had once passed through an attack of the *icteroides* infection were absolutely immune to a second infection with the same organism but reacted severely and sometimes fatally to a later inoculation of *icterohæmorrhagiæ*. Although there were a number of instances in which a previous infection with *icteroides* did not confer any perceptible immunity upon the guinea pigs against *icterohæmorrhagiæ*, another group of guinea pigs showed a considerable resistance to the *icterohæmorrhagiæ* infection as compared with those which had never been inoculated with *icteroides*. There is not much doubt, therefore, that an *icteroides* attack brings about, in some instances at least, a certain degree of resistance to the *icterohæmorrhagiæ* infection. Hence the study of the phenomena of active immunity strongly indicates that *icteroides* is closely related immunologically to *icterohæmorrhagiæ*.

ETIOLOGY OF YELLOW FEVER.

XI. SERUM TREATMENT OF ANIMALS INFECTED WITH *LEPTOSPIRA* ICTEROIDES.

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The high potency attained by a polyvalent immune serum for *Leptospira icteroides* derived from the horse, as revealed in previous experiments on guinea pigs,¹ indicated the possibility that such a serum might be advantageously employed in the treatment of patients suffering from an infection with that organism. In order to ascertain whether or not the serum can exert a beneficial influence upon the course of the infection, several series of experiments were planned in which the guinea pigs were first inoculated with multiple minimal lethal doses of *Leptospira icteroides* culture and then treated with the immune serum at varying intervals afterwards.

Emphasis has been laid upon the fact that guinea pigs vary considerably in their susceptibility to *Leptospira icteroides*,² and instances have been cited¹ in which some animals survived after the injection of a large amount of culture, while some succumbed to smaller amounts. Irregularities of this nature were to be anticipated in the present series of experiments, but this source of error was eliminated as much as possible by using the serum in several graduated doses on a corresponding number of animals for a number of days in succession. In some of the earlier series tests were rendered unsatisfactory by the use of a culture which, from unknown causes, failed to kill the control animals, notwithstanding the fact that the same culture had been highly virulent when tested a fortnight previously. In another series the virulence of the culture employed was such that the control

¹ Noguchi, H., *J. Exp. Med.*, 1920, xxxi, 135.

² Noguchi, H., *J. Exp. Med.*, 1919, xxix, 585.

guinea pigs died within 5 or 6 days after the injection of the culture, and the time for treatment was comparatively brief.

The serum was injected intraperitoneally in amounts of 0.001, 0.01, 0.1, and 1 cc. at intervals of 1, 24, 48, 72, and 96 hours, and even 5, 6, and 7 days, if the animals still lived, after the inoculation of the culture. In the earlier experiments two injections a day of each dose were given (10 a.m. and 4 p.m.), but this practice was soon abandoned in favor of a single daily injection.

In determining the effect of the immune serum upon the infection, the temperature and other characteristic clinical symptoms were noted, and the extent of the lesions was ascertained by killing the surviving animals at a later period, when they were regarded as convalescent. The lesions chiefly considered, although not a wholly reliable index of the severity of the infection, were the hemorrhagic foci in the lungs.

In a series of therapeutic experiments in which the control guinea pigs died within 5 to 6 days, a culture of Strain 5 of *Leptospira icteroides* being employed, it was found that when injections of the immune serum were begun within 24 hours from the time of the experimental infection (intraperitoneal), no symptoms or lesions developed, provided the animal had received more than 0.001 cc. of the serum. If the treatment was begun 48 hours after infection, it was necessary to inject more than 0.01 cc. in order to prevent their development. At the end of 72 hours, when some of the guinea pigs had begun to show a rise of temperature, more than 0.1 cc. of the serum was necessary to check the progress of the infection, although some animals treated with 0.01 cc. also recovered. After 96 hours, when most of the animals had a high temperature, and some had begun to show a trace of jaundice, the injection of more than 0.01 cc. had a marked influence. The temperature came down to 100–102°F. by the following morning, and no jaundice afterwards appeared in these animals, which recovered within a week. A few guinea pigs, which were slightly icteric at the time of injection of the serum, became more deeply jaundiced on the following day and remained so for a few days, when they began to convalesce. No guinea pig treated within 96 hours with more than 0.01 cc. of serum died.

At the end of 5 days most of the animals which had not been treated with the serum showed jaundice and a decline in temperature, signs of approaching death within 24 hours. A few animals died in the afternoon of that day. To several of these critically ill guinea pigs 0.1 to 1 cc. of the serum was given twice, at 10 a.m. and at 4 p.m., but none of them seemed to be influenced by the injection. Some died on the same day, others on the following day, showing all the typical symptoms. At the end of 6 days several animals which still remained alive without the serum were treated with two doses of 1 cc., but none was saved from death on the same day.

It was found from this series of experiments that the progress of jaundice, after it had existed for 24 hours, could not be checked by the injection of the immune serum.

All the surviving animals were later killed for examination of the lung lesions. The animals treated with a sufficient amount (0.001 cc. or more) within 24 hours showed no lesions, or at most only a few hemorrhagic spots in the lungs. On the other hand, fairly numerous old hemorrhagic foci were found in the lungs of those which had been treated at the end of 48 hours, indicating an infection aborted through the action of the serum. The lung lesions were decidedly more diffuse and numerous in the guinea pigs which received the serum at the end of 72 and 96 hours, although none succumbed to the infection. In the animals which died following treatment with the serum later than 5 days after infection the symptoms and lesions were typical of the infection and indistinguishable from those in the control animals which died without receiving any serum.

In Table I is given another series of experiments carried out with Strain 6 of *Leptospira icteroides*. This culture killed control animals in doses of 0.001, 0.01, and 0.1 cc. within 13, 10, and 7 days respectively, although one guinea pig inoculated with 1 cc. of the culture escaped death after a severe infection. The amount of culture employed throughout the series was 0.5 cc. and was given intraperitoneally. The injections of serum were begun 1 hour after the inoculation and repeated daily for 7 days, in doses of 0.01, 0.1, and 1 cc.

As the table shows, no infection, so far as external manifestations are concerned, took place in any of the guinea pigs injected with the

TABLE I
Effect of Polyvalent Immune Serum on Experimental Infection with *Lepidoptera leishanensis*

June 14, 1919. Thirty-seven guinea pigs were inoculated intraperitoneally, each with 0.5 cc. of Strain 6 of *Lepidoptera leishanensis* culture, representing at least 300 individual infected donors. Graded quantities (0.01, 0.1, and 1 cc.) of Immune Bence Serum 2 were given to the inoculated guinea pigs at intervals of 1, 2, 4, 7, and 96 hours, etc. The object of the experiment was to ascertain the minimal amount of the immune serum which prevents a fatal infection and the time limit within which the inoculated animals may still be saved by the injection of the serum.

Guinea pig No.	Time elapsed after infection	Amount of serum injected	Conditions preceding infection of immune serum	Subsequent course of infection (after infection of immune serum)	Remarks
s101a	4	0.01	No noticeable effects from infection	No symptoms	No leishana found
s101b	1	0.1	"	"	"
s103a	1	1.0	"	"	"
s103b	91	0.01	"	"	Several old hemorrhages in lungs
s106a	94	0.1	"	"	A few minute foci of old hemorrhages in lungs
s106b	94	1.0	"	"	No leishana found
s107a	193	0.01	"	"	Further diffuse old hemorrhages in lungs
s107b	193	0.1	"	"	No leishana found
s108a	193	1.0	"	"	A few old hemorrhages foci in lungs
s108b	73	0.01	"	"	Several old hemorrhages foci in lungs
s109a	72	0.1	"	"	No leishana found
s109b	72	1.0	"	"	A few old hemorrhages spots in lungs

x _{100a}	96	0 01	Temperature 104°F. in a.m. but animal not apparently sick; no jaundice. 1st day of disease (4 day incubation period). Serum given in p.m.	Temperature 105.5°F. after injection of serum. Following a.m. 102.5°, 101.5° in p.m. No further symptoms.	A few old hemorrhagic foci in lungs.
x _{100b}	96	0 1	Temperature 101.5° in a.m.; no other symptoms. Serum given in p.m.	Temperature 104.5° in p.m.; normal next day. Animal well thereafter.	Numerous old hemorrhagic foci in lungs.
x _{101a}	96	1 0	Animal remained well (temperature 102°) after injection of serum.	No change in temperature (102.5°). Animal remained well throughout experiment.	No lesions found.
x _{101b}	120	0 01	Had had high temperature (104°, 103°) 2 preceding afternoons. Temperature not high in a.m. when serum was given (101.5°).	Temperature 103.5° in p.m.; animal, however, seemed well. No further symptoms. Temperature returned to normal following day. Survived.	" " (natural refractoriness suspected).
x _{102a}	120	0 1	Fever since preceding day (103.5-104°). Temperature in a.m. on serum injection 103°; rose to 104° in p.m.	Temperature next day 103.5° in a.m. and 104.5° in p.m., but on following a.m. 101.5°. Afternoon rise to 104° for 2 more days, then return to normal. Animal survived.	Numerous pale hemorrhagic foci in lungs.
x _{102b}	120	1 0	Temperature 104° in a.m. and 105.5° in p.m. of day before. 101.5° in a.m. of day serum was given in p.m. Jaundice not distinct.	Temperature remained 104.5° in p.m. 102° next a.m. and 101° in p.m. No further febrile or other symptoms. No jaundice developed. Survived.	Pale hemorrhagic foci in lungs.

* All the guinea pigs surviving were killed on July 4, 1919 (21 days after infection) for examination.

TABLE I—Continued.

Guinea pig No.	Time elapsed after inoculation.	Amount of serum injected.	Conditions preceding injection of immune serum.	Subsequent course of infection (after injection of immune serum).	Remarks.*
	days	cc.			
x ₁ 103a	6	0.01	Fever for 3 preceding days (103.5°, 104°, 104.5°). 102° in a.m. of serum injection. Distinct jaundice; animal very weak, bordering on collapse.	Temperature rose to 104° in p.m. after serum injection. Animal gradually became worse, dying 9 days after infection.	<i>Autopsy</i> .—Extreme jaundice, and general hemorrhage, especially in lungs.
x ₁ 103b	6	0.1	Fever for 3 preceding days (103°, 104.5°, 104.5°). Temperature 104° in a.m. of injection. Slight trace of jaundice.	Temperature 104.5° in p.m. after injection of serum. Fever disappeared next day. Animal well throughout remainder of experiment. Survived.	Extensive foci of old hemorrhages in lungs.
x ₁ 104a	6	1.0	Fever for 3 preceding days (104.5°, 106°, 105°). 103° in a.m. of serum injection. Mild jaundice.	Temperature same p.m. 104.5°, but only 101° next day. Jaundice persisted 3 days longer, then faded. Survived.	Extensive foci of old hemorrhages in lungs.
x ₁ 104b	7	0.01	Fever for 2 preceding days (105°, 102.5°). Temperature 104° on day of injection. Jaundice slight.	Temperature 104° after serum injection, but 102° next 3 days, returning slowly to normal. Jaundice disappeared in few days.	Rather numerous old hemorrhagic lesions in lungs.
x ₁ 106a	7	0.1	Fever 3 preceding days (106°, 104.5°, 103.5°). Temperature 102.5° in a.m. of serum injection. Distinct jaundice. Serum given in p.m.	Temperature 103.5° same p.m., 102.5° next a.m., 103.5° p.m., 102° next a.m., 101° p.m. Jaundice persisted 3 days, then faded. Survived.	Very marked hemorrhagic lesions in lungs.

x107a	7	1.0	Fever 3 preceding days (105°, 104.5°, 103°). Temperature 102° in a.m. of serum injection. Marked jaundice. Serum given in p.m.	Temperature 103.5° in p.m.; 101-101.5° on days following. Jaundice increased for 2 days after injection of serum, disappearing 5 days later. Survived. Temperature 103.5° same p.m.; 100° next a.m. Jaundice and collapse. Died 8 days after inoculation.	Hemorrhagic foci in lungs less marked than in preceding animal. <i>Autopsy.</i> —Typical lesions.
x107b	7	1.0	High temperature for 4 afternoons preceding (104°, 104°, 103°, 104°), but less in mornings (102.5°, 104°, 102°, 102°). Temperature 102.5° in a.m. of serum injection. Jaundice intense for 2 days. Serum given in p.m.		

Controls.

Guinea pig No.	Amount of culture.	Course of infection.	Remarks.*
x192a (control).	0.001	Incubation of 9 days, followed by fever for 4 days (104°, 102°, 105.5°, 103°F.). Jaundice noticed on 3rd day of disease; increased in intensity for 3 days following. Death occurred, with temperature 98°, 13 days after inoculation.	<i>Autopsy.</i> —Typical lesions.
x192b (control).	0.01	Incubation of 4 days, followed by period of fever for 5 days. Jaundice developed in 7 days and became extremely intense within 24 hrs., remaining so until death, which occurred on 10th day after inoculation, with temperature of 96.5°.	" " "
x193a (control).	0.1	Incubation period 4 days, followed by fever for 48 hrs. (104.5°, 103.5°), then collapse, with temperature 99° on 8th day. Jaundice appeared on day before death, which occurred 7 days after inoculation.	" " "
x193b (control).	1.0	Temperature 103.5° on 4th day after inoculation. Fluctuated for 48 hrs. longer, but was at no time higher. Jaundice appeared on 6th day, increased in intensity rapidly during next 48 hrs., then receded, having disappeared 4 days later. Animal survived.	Killed. Extensive hemorrhagic lesions in lungs.

serum within a period of 72 hours from the time of inoculation with the culture. All remained well.

At the end of 96 hours most of the animals had a temperature of 103-104.5°F. in the morning. The injections of the serum were made in the afternoon. The temperature remained high after the injection but dropped gradually the following day, and the animals recovered rapidly.

At the end of 5 days most of the animals had had a high fever for 2 days. The serum was given in the afternoon. Two of the three guinea pigs treated began to improve the next day, one had a high temperature for a day longer, but all eventually returned to the normal condition.

At the end of 6 days the animals had begun to show more or less jaundice and had had fever for 3 days. One guinea pig which received 0.01 cc. of the serum died 9 days from the time of infection, while those which received 0.1 and 1 cc. recovered.

Experiments were made at the end of 7 days, when the animals had had fever for about 3 days, and a slight decline had begun in the morning. In some there had been jaundice for 48 hours. The serum was injected in the afternoon. The temperature the following morning was quite high in most animals, and in some it lasted for 2 to 3 days, while in others it went down rapidly. Jaundice increased in intensity in some, but gradually disappeared within several days. All recovered except one, which was near collapse when 1 cc. of serum was injected and which died the next day.

Of the surviving guinea pigs, no lesions were found in the lungs of those treated with the serum within 1 hour; and in each group of three guinea pigs treated 24, 48, 72, or even as late as 96 hours (during the incubation period) there was one animal in which no lesion was present. In others, irrespective of the amount of serum given, there were a certain number of hemorrhagic spots in the lungs, even when treated with the serum within 24 hours. In one instance there was no lung lesion in a guinea pig treated with 0.01 cc. after 5 days. These irregularities are explained by the existence of a considerable variation among individual animals in their susceptibility to *icteroides* infection.

On the whole, the results obtained in this series admit of only one interpretation; namely, that the immune serum, when injected during the period of incubation, prevents further development of the infection, and that when used in the early stage of the disease it is capable of preventing a fatal termination of the infection. On the other hand, when guinea pigs are inoculated with a highly virulent culture, the injection of the serum at a period of the disease when jaundice has existed for some time and the animal is nearing collapse seems to have no benefit. Undoubtedly the virulence of the strain employed for the experiments has considerable bearing upon the efficacy with which the immune serum may be used in the late stage of the disease. The less virulent the culture, the greater are the chances of benefit from the injection of the serum after the clinical symptoms have manifested themselves, as shown by the favorable results obtained in the last series of experiments just recorded. The fact must be emphasized, however, that even with a less virulent strain the serum has no beneficial effect when given to a guinea pig which is in a condition bordering on collapse (fall of temperature, with intense jaundice).

SUMMARY.

The use of a polyvalent immune serum of high potency in the treatment of an experimental infection of guinea pigs with *Leptospira icteroides* was found to be of definite advantage in checking the progress of the infection. When administered during the period of incubation the serum was found capable of completely preventing the development of the disease, although on subsequent examination hemorrhagic lesions of greater or less number and extent were found in the lungs of the guinea pigs which survived. Moreover, the serum modified the course of the disease and when used in the early stages of infection prevented a fatal outcome. Employed at a later stage, however, when jaundice and nephritis had been present for several days and the animal was near collapse, the serum had no perceptible beneficial effect. This was, of course, to be expected in view of the incidence of various pathological phases of this disease—nephritis, hepatitis, and other toxic symptoms in succession. In man the clinical manifestations are more gradual and distinct than in the

guinea pig,³ yet the yellow fever patient whose temperature is subnormal, and who has reached the stage of hemorrhages from the gums, nose, stomach, and intestines, and of uremia and cholemia, would seem to have little or no chance of deriving benefit from the use of a specific immune serum. This latter assumption would probably hold irrespective of the relation which *Leptospira icteroides* proves to have to the etiology of yellow fever.

³ Noguchi, H., *J. Exp. Med.*, 1919, xxix, 547.

THE STERILIZATION OF LIPOVACCINES.

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The proposal to substitute suspensions of bacterial bodies in oil for suspensions in saline solution as a vaccine for use in combating typhoid fever and pneumonia theoretically offers great advantages. Provided the vaccination is safe and efficient, it would be of great importance to be able to get the same result with a single application that has, in the past, been accomplished with three doses of the prophylactic.

The evidence so far at hand would seem to show that the vaccination with oily suspensions causes a formation of antibodies comparable with that afforded by suspensions in water, and thus renders it probable that a similar degree of protection may be given by the lipovaccine. The matter is, however, of sufficient importance to justify repeated examination.

When material of this nature is to be widely distributed its sterility should be assured both by the method of preparation and control tests on the finished product. In this respect the methods proposed by Whitmore and Fennel¹ are not altogether satisfactory. The procedures recommended afford many opportunities for contamination, the oil interferes with the action of antiseptics so that these cannot be depended upon for a final sterilization, and lastly it is far from easy to make satisfactory tests for sterility on the finished product.

Rosenow and Osterberg,² recognizing these difficulties, propose to kill the bacteria and any contaminating organisms with a water solution of an antiseptic, to form an emulsion with the oil, and finally

¹ Whitmore, E. R., and Fennel, E. A., *J. Am. Med. Assn.*, 1918, lxx, 902.

² Rosenow, E. C., and Osterberg, A. E., *J. Am. Med. Assn.*, 1919, lxxiii, 87.

to remove the water by vacuum distillation at a comparatively low temperature. This appears from their statement to be an efficient method of preparation.

In approaching this matter we have followed another line of thought and wish to record our results at this time as a contribution to the subject without, however, attempting to decide which method may in the end be best.

Loeffler³ has stated that by the application of dry heat bacteria may be killed without destroying their antigenic properties. The temperature he used was 70°C. and the exposure was prolonged for days or weeks. It is known that glassware and fabrics may be sterilized by exposure to dry heat for a number of hours, the length of exposure varying inversely with the temperature. Thus 130°C. for 3 hours is as efficient as 160°C. for 1 hour. The suspensions of bacteria in oil are essentially dry preparations, and it seemed possible that the finished vaccine could be sterilized at an intermediate temperature without destroying its antigenic qualities. As a matter of fact, our results indicate that this may be accomplished with pneumococcus. We have so far been unsuccessful with *Bacillus typhosus*.

EXPERIMENTAL.

Pneumococcus lipovaccine was prepared according to the method of Whitmore and Fennel.¹ Briefly, the bacteria were grown in glucose broth and separated by centrifugalization after about 18 hours growth. The bacterial mass was dried in an oven at 53°C. over lime. The dry mass was ground in a Pyrex jar with steel balls for a number of hours, a mixture of anhydrous lanolin and cottonseed oil (Wesson) added, and the grinding continued. Finally, sterile cottonseed oil containing 0.25 per cent chloretone was added in such amount that each cubic centimeter of the finished product would contain 2 mg. of the dried bacterial powder. The grinding was then continued for a number of hours, the product put into suitable bottles or ampules and sealed as ready for distribution.

Control tests for sterility revealed the presence of *Bacillus subtilis* and sometimes other bacteria in the dried mass in a majority of

³ Loeffler, F., *Deutsch. med. Woch.*, 1913, xxxix, 1025.

instances. When the dried bacterial mass was clean the finished product was nearly always sterile. The pneumococcus does not survive the drying process. If the dried mass containing *Bacillus subtilis* is ground in oil containing chloretone this contaminating organism will remain viable for months. With experience in manipulation the amount of material lost because of contamination was reduced, but even with the greatest care it always remained high.

In an attempt to overcome this difficulty we exposed the finished vaccine to a temperature of 130°C. for 3 hours in an electric oven (Freas), and to 120°C. for 12 hours, temperatures which are sufficiently high to sterilize any material. The vaccine, heated and unheated, was administered to healthy mice (0.5 cc. subcutaneously), and after varying periods the resistance of the mice to pneumococcus infection was tested by intraperitoneal injection of doses of culture known to be fatal, or multiples thereof. The results of two experiments are given in Tables I and II.

The experiments show that a considerable degree of protection is afforded to mice by the administration of pneumococcus lipovaccine. In the occasional instances in which the vaccinated mice died it was found that the vaccine had not been absorbed, almost the whole mass injected remaining in the subcutaneous tissues. Protection is exerted against at least ten fatal doses of culture. The mice in the experiments reported had been treated with vaccine 35 and 38 days previously. In other experiments in which the interval was 21, 56, and 110 days, there was no evidence of protection, indicating that the immunity following a single dose of lipovaccine is slow to develop and is transient. The heat treatment did not decrease the antigenic qualities of the vaccine appreciably.

The administration of vaccine in practice as a prophylactic against pneumonia is still in the stage of trial. Since at present the qualities of typhoid vaccine are of much greater interest, we have extended our work to include this phase of the subject.

Typhoid lipovaccine⁴ similarly heated and unheated was administered to rabbits intraperitoneally, a single dose of 1 cc. being given. The blood was tested for its agglutinin content at intervals there-

⁴ The vaccines used in this experiment were obtained from the U. S. Army Medical School through the courtesy of Major H. J. Nichols.

TABLE I.

Protection Test of Mice Treated with Pneumococcus Lipovaccine.

The vaccinating dose was administered 35 days before the test. The test consisted of inoculation with the indicated quantity of a 24 hour bouillon culture injected intraperitoneally with normal salt solution sufficient to make a total volume of 0.5 cc.

Vaccine.	Culture.		Result.			
	Type.	Dilution.	After 24 hrs.	After 48 hrs.	After 72 hrs.	After 96 hrs.
None.....	III	1:10,000,000	Sick.	Sick.	Dead.	
	III	1:10,000,000	"	Dead.		
	III	1:100,000,000	"	"		
	III	1:100,000,000	Dead.			
	I	1:10,000,000	Sick.	Sick.	Dead.	
	I	1:10,000,000	Dead.			
	I	1:100,000,000	Sick.	Dead.		
	I	1:100,000,000	"	"		
Unheated.....	III	1:10,000,000	"	"		
	III	1:10,000,000	"	Sick.		Well.
	III	1:100,000,000	"	"		"
	III	1:100,000,000	Dead.			
	I	1:10,000,000	Sick.	Sick.		Well.
	I	1:10,000,000	Dead.			
	I	1:100,000,000	Sick.	Sick.		Well.
	I	1:100,000,000	Dead.			
Heated to 130°C. for 3 hrs. . .	III	1:10,000,000	Sick.	Sick.		Well.
	III	1:10,000,000	"	"	Dead.	
	III	1:100,000,000	"	"		Well.
	III	1:100,000,000	"	"		"
	I	1:10,000,000	"	"		"
	I	1:10,000,000	"	"		"
	I	1:100,000,000	"	"		"
	I	1:100,000,000	"	"		"
Heated to 120°C. for 12 hrs. .	III	1:10,000,000	"	"		"
	III	1:10,000,000	"	"		"
	III	1:100,000,000	"	"		"
	III	1:100,000,000	"	"		"
	I	1:10,000,000	"	Dead.		
	I	1:10,000,000	"	Sick.		Well.
	I	1:100,000,000	"	"		"
	I	1:100,000,000	"	"		"

TABLE II.

Protection Test of Mice Treated with Pneumococcus Lipovaccine.

The vaccinating dose was given 38 days before the test. The test consisted of inoculation with the indicated quantity of a 24 hour bouillon culture of Type I pneumococcus intraperitoneally. The total volume injected was 0.5 cc.

Vaccine.	Dilution of culture.	Result.	
		After 24 hrs.	After 48 hrs.
None.....	1:1,000,000	Sick.	Dead.
	1:1,000,000	"	"
	1:1,000,000	Dead.	
	1:1,000,000	Sick.	Dead.
	1:1,000,000	"	"
	1:1,000,000	Dead.	
	1:1,000,000	Sick.	Dead.
	1:1,000,000	Dead.	
Unheated.....	1:1,000,000	Well.	Well.
	1:1,000,000	"	"
	1:1,000,000	"	"
	1:1,000,000	"	"
	1:1,000,000	Sick.	"
	1:1,000,000	"	"
	1:1,000,000	"	Dead.
	1:1,000,000	"	Well.
Heated to 130°C. for 3 hrs.....	1:1,000,000	"	"
	1:1,000,000	"	"
	1:1,000,000	"	"
	1:1,000,000	"	"
	1:1,000,000	"	"
	1:1,000,000	"	Dead.

after. As a control the results were compared with those obtained by the administration of three doses of typhoid vaccine in saline suspension intraperitoneally at 5 day intervals. The results are presented in Table III.

The experiment shows that at least for these particular preparations the lipovaccine is less efficient in single doses than is the saline preparation in three doses. The antigenic qualities of the lipovaccine (typhoid) are almost destroyed by heating to 130°C. for 3 hours.

TABLE III.
Agglutinin Production in Rabbits Treated with Typhoid Vaccines.

Rabbit No.	Interval since last dose, days	Vaccine.	Serum dilution.								Remarks.
			1:20	1:40	1:100	1:150	1:200	1:500	1:1,000	1:2,000	
1	16	Saline.	++	++	++	++	++	++	++	++	27 days after first dose.
2	16	"	++	++	++	++	++	++	++	+	
3	16	"	++	++	++	++	++	++	++	0	
4	16	"	++	++	++	++	++	++	+	0	
5	17	Lipovaccine I	++	++	++	0					Rabbits tested 12 days later show no increase in agglutinins.
6	17	"	++	++	++	++	++	0			
7	17	"	++	++	++	++	++	0			
8	17	"	++	++	++	0					
9	17	"	++	++	0						
10	17	"	0	0	0	0					
11	17	"	0	0	0	0					
12	17	"	++	++	++	0					
Lipovaccine heated to 130°C. for 3 hrs.											
13	16	Lipovaccine I	++	0	0						
14	16	"	++	+	0						
15	16	"	++	++	0						
16	16	"	+	0	0						
17	16	"	++	++	0						
18	16	"	0	0	0						
19	16	"	++	++	0						
20	16	"	0	0	0						

SUMMARY.

Pneumococcus lipovaccine confers a definite protection against pneumococcus infection in mice. The protective quality is not destroyed, and apparently is not greatly diminished, by heating to 130°C. for 3 hours or 120°C. for 12 hours.

Typhoid lipovaccine gives rise to the formation of agglutinins in rabbits but to a lesser degree than saline suspensions. The antigenic qualities of the typhoid lipovaccine are greatly injured by heating to 130°C. for 3 hours.

COAGULATION IN EMBRYONIC BLOOD.*

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INTRODUCTION.

In the course of an experimental study of the origin of non-nucleated erythrocytes (Emmel (1)), certain observations were made indicating a slower coagulation time and also other striking differences in the reaction of embryonic blood as compared with that of adult blood. The present work represents a more extended study of this subject with the purpose of ascertaining (1) the facts regarding the coagulation time of embryonic blood; (2) to what extent the factors essential to coagulation are comparable in embryo and adult; and (3) whether any of the conditions found in the embryonic blood may be of possible significance with reference to certain types of abnormal coagulation occurring in postnatal life. With but one exception (Boll (2)) there appear to be no data concerning coagulation in embryonic blood recorded in the literature.

Coagulation Process in Normal Embryonic Blood.

Material and Technique.

The present study of coagulation is largely confined to pig embryos 100 to 270 mm. in length. The material was obtained from the uterus under favorable circumstances within a short interval after the killing of the parent.¹ Only embryos in apparently perfectly normal condition were used, in which the hearts were still beating or would, in any

* Presented at the thirty-fifth session of the American Association of Anatomists, Pittsburgh, April 17-19, 1919.

¹ This material was obtained through the courtesy of Swift and Co.

event, respond to stimulation—a point of considerable importance, since it was found that in the blood from embryos in which the hearts were not beating the coagulation time was materially reduced.

In each instance the umbilical cord was ligated and cut and the embryo removed from the uterus. Part of the anterior thoracic wall was then cut away, the pericardial sac opened, and the heart exposed. The needle (platinum) of a graduated hypodermic syringe was immediately inserted into the left ventricle and care taken to obtain the blood without air bubbles or tissue juices. This blood was then transferred in 0.5 cc. quantities to a series of test-tubes, again with care against the introduction of air.

In general the technique used is in accordance with that of Lee and White (3). Test-tubes of various sizes were tried, but the best results were obtained with tubes having an inside diameter of 9 mm. and a length of about 9 to 10 cm. The glassware and syringes, thoroughly cleaned in chromic acid, were rinsed with isotonic salt solution previous to use in every experiment. The coagulation of the blood was determined by tilting the test-tube at short intervals and noting the formation of a sliding clot or gel, sufficient in the latter case to withstand the complete inversion of the tube. These experiments were made at a room temperature of about 21°C.

Coagulation Time and Character of the Clot.

Table I presents the data for the coagulation time of the blood obtained in eighteen embryos of various sizes. These results are typical of about 50 different experiments and observations. In these data it will be observed that the average normal coagulation time for the blood of the embryos is 23.3 minutes.

This is in marked contrast to the results shown in Table II which were obtained for the adult pig. The blood had been allowed to flow into a clean receptacle as it escaped from the cut carotid and jugular vessels of the animal, and the coagulation time determined for oxalated and non-oxalated blood by the same technique as previously described for the embryo.

On the basis of these data it is evident that the coagulation time for embryonic blood is greatly delayed as compared with that of adult blood, the coagulation time for the embryo being about six to eight times greater than that of the adult. In this connection it may be observed that the coagulation time obtained here for the adult blood may be slightly below normal due to some contact with tissue sur-

faces as the blood gushes from the cut vessels, although this does not seem to be sufficient to affect materially our conclusions.

Certain points are to be noted regarding the process of coagulation as observed in these experiments on embryonic blood. The normal

TABLE I.

Normal Coagulation Time for Embryonic Blood.

Size of embryo.	Volume of blood.	Length of time before first evidence of fibrin was observed.	Length of time for formation of clot.	Size of embryo.	Volume of blood.	Length of time before first evidence of fibrin was observed.	Length of time for formation of clot.
<i>mm.</i>	<i>cc.</i>	<i>min.</i>	<i>min.</i>	<i>mm.</i>	<i>cc.</i>	<i>min.</i>	<i>min.</i>
100	0.5	25	30	190	0.5	17	23
120	0.5	19	28	200	0.5	19	22
130	0.5	24	30	210	0.5	12	21
135	0.5	18	30	220	0.5	12	24
140	0.5	15	21	240	0.5	18	21
150	0.5	12	22	240	0.5	17	21
160	0.5	12	20	250	0.5	17	24
170	0.5	12	21	250	0.5	17	21
180	0.5	16	20	270	0.5	12	20
Average						16.3	23.3

TABLE II.

Normal Coagulation Time for Adult Blood.

Non-oxalated blood.		Oxalated blood.*		
Volume of blood.	Coagulation time.	Volume of blood.	CaCl ₂ 0.5 %.	Coagulation time.
<i>cc.</i>	<i>min.</i>	<i>cc.</i>	<i>gt.</i>	<i>min.</i>
0.5	3	0.5	1	4
0.5	3	0.5	2	4
0.5	4	0.5	3	3
0.5	3	0.5	3	4

* A 1 per cent solution of sodium oxalate was used in the proportion of one part of oxalate to eight parts of blood.

clot never attains a density or firmness comparable with that of adult blood. As a rule, the clot is like a mass of semisolid gel, which at its maximum is usually equal in volume to about two-thirds the total quantity of blood. Since this mass or lump may be made to

move back and forth by tilting the tube, it may be appropriately described as a sliding clot. In contrast to the clot of the adult this clot is seldom of sufficient firmness to permit a complete inversion of the tube. Not infrequently the sliding clot is very small, if not entirely absent.

The first indication of coagulation to be observed consists in the appearance of small particles or clumps of fibrin which are almost invariably deposited at the side of the tube. The formation of a complete gel or clot occurs within an average of about 7 minutes after the first appearance of fibrin, as shown in Table I. Within a short time (30 to 50 minutes) this gel retracts into a small compact mass. If the blood is not too greatly disturbed, the initial deposit of fibrin seems to form a nucleus around which the entire mass of fibrin accumulates during clot retraction. In some instances it appears that the total quantity of fibrin may be deposited in a compact mass without the formation of an intermediate gel stage and this accounts for the occasional absence of a sliding clot.

The data indicate some reduction in coagulation time in the oldest embryos, although the differences apparently are not pronounced within the periods of development to which the present study is confined. In this connection it is to be taken into account that size measurements alone cannot always be taken as an absolute index of the age and degree of embryonic development, since embryos taken from the same uterus may differ by as much as 30 to 40 mm. in length.

Analysis of Possible Factors Involved in the Greater Coagulation Time of Embryonic Blood.

The coagulation of blood is primarily dependent upon the interaction of three factors, prothrombin, calcium, and fibrinogen. Since the coagulation of embryonic blood is, as has just been demonstrated, much slower than that of adult blood, the question arises whether these factors are equally active in the embryo or whether other conditions are to be taken into account. The following analysis has been made with the view of obtaining data toward the solution of this problem.

Blood Platelets.

Platelet Content.—Of the fundamental factors of coagulation, one, the prothrombin, is evidently chiefly a derivative of blood platelets. In many cases of pathological hemorrhage, such as hemophilia and purpura hæmorrhagica, the abnormal variations in coagulation are found to be primarily related to quantitative or qualitative deficiencies in this platelet material. Attention was consequently first directed to the question of the platelet content of the embryonic blood.

From an examination of properly stained preparations of the blood from pig embryos it is obvious that platelets are present in great numbers. Jordan (4) has recently shown that blood platelets are abundant even in 12 mm. pig embryos. A quantitative estimation of these elements based upon numerical data is presented in Table III.

TABLE III.
Platelet Count for Embryonic and Adult Blood.

Size of embryo.	Average No. per c.mm.	Size of embryo.	Average No. per c.mm.
<i>mm.</i>		<i>mm.</i>	
25	340,000	120	464,000
40	448,000	140	422,000
45	296,000	270	800,000
95	415,000	Adult.	588,000*

* Derived from a number of counts in which the platelet content varied from 544,000 to 932,000.

In making the platelet count, 3 per cent sodium citrate was used, in accordance with the method described by Ottenberg and Rosenthal (5). The averages are based upon three or more counts in each instance. The platelet counts show a surprising number of platelets in the embryonic blood. With the exception of certain instances, as in the case of the 270 mm. embryo, the platelet count is apparently somewhat less than that of the adult. This difference, however, does not appear sufficient to account for the delay in the coagulation of the embryonic blood. With human blood, averaging about 250,000 platelets per c.mm., pathological hemorrhage does not occur until the platelet count falls below 60,000 (Minot (6)²). This represents

² Minot (6), p. 1105.

a drop of 75 per cent in man, whereas in no instances in pig embryos between 100 and 270 mm. did the platelet count fall below 27 per cent of the average for the adult.

Coagulation Reaction after the Addition of Platelet Preparations from Adult Blood.—In attempting to determine to what extent blood platelets may be involved in abnormal coagulation of blood, it is evident that a quantitative determination of the platelet content is not necessarily sufficient, as in the case of hemophilia, for example, where the platelets may be present in approximately normal number but still be abnormal in their activity (Lee and Minot (7)³). In view of this possibility the following experiments were made in which preparations

TABLE IV.

Effect of the Addition of Adult Platelet Material on the Coagulation Time of Embryonic Blood.

Size of embryo.	Volume of blood.	Coagulation time.		Size of embryo.	Volume of blood.	Coagulation time.	
		Normal control.	Blood plus 2 drops of platelet suspension.			Normal control.	Blood plus 2 drops of platelet suspension.
mm.	cc.	min.	min.	mm.	cc.	min.	min.
100	0.5	30	10	200	0.5	22	10
130	0.5	30	5	220	0.5	24	8
140	0.5	21	7	240	0.5	21	11
160	0.5	20	13	250	0.5	21	12
180	0.5	20	4	270	0.5	20	4
Average						23	8.4

of platelet material from adult pig blood were added to embryonic blood with the results given in Table IV. The suspension of platelet material was prepared in accordance with the technique of Le Sourd and Pagniez (8).

The blood, caught in clean receptacles as it escaped from the vessels of the animal, was immediately transferred to 50 cc. centrifuge tubes and mixed with equal volumes of a solution of 0.4 per cent sodium oxalate in isotonic saline solution, the final mixture of blood with the solution containing 0.2 per cent sodium oxalate. These tubes were then centrifuged at 1,500 revolutions per minute for 10 to 15 minutes and a little more than the upper half of the clear plasma was pipetted off

³ Lee and Minot (7), p. 80.

and again centrifuged at 1,500 revolutions for 15 minutes longer to throw down any remaining cells. This oxalated plasma was then centrifuged in 15 cc. centrifuge tubes for $\frac{1}{2}$ hour at 3,000 revolutions per minute. The clear supernatant plasma was then decanted, leaving a small sticky grayish white mass of platelet material at the bottom of the tube. A quantity of a solution of 0.2 per cent sodium oxalate in isotonic saline solution was then added to each tube, the platelets were stirred up in this fluid, and the resulting suspensions from four tubes collected into one tube and again centrifuged for 15 minutes at 3,000 revolutions. The supernatant fluid was poured off and the platelets from the entire volume of blood (200 cc.) were extracted with 2 cc. of distilled water. The resultant milky white suspension of platelets was used for experimental purposes within 24 hours after preparation.

These data demonstrate striking results upon the addition of platelet suspensions. The coagulation time of 23 minutes for the normal controls was reduced to 8.4 minutes by the addition of platelet material—a reduction of nearly 75 per cent. The process of coagulation was also attended by the formation of a larger and more homogeneous clot or gel. Not only are these results of interest in that the coagulation time can be materially shortened, but they seemed for the moment to emphasize some abnormal activity of the blood platelets or prothrombin as a factor in the greater coagulation time of embryonic blood. But, as will develop later, it was found that this conclusion could not be maintained.

Examination of these results shows that the effect of the addition of platelet material is by no means constant. In some instances the blood coagulated in 12 to 13 minutes, whereas in others the clot was formed in 4 minutes—a period equivalent to the adult coagulation time. This variation may be due, in part, to qualitative differences in the platelet preparation, for the suspensions differed in opacities, and, as emphasized by Lee and Vincent (9),⁴ it is difficult to obtain platelet suspensions of constant strength. Platelet material alone was not sufficient to account for the marked degree of variation in the present results. This raised the question as to whether other factors may be involved in the problem. That these results cannot be due to mere dilution of the blood was shown conclusively by tests with isotonic salt solution and distilled water in which the results were negative.

⁴ Lee and Vincent (9), p. 407.

Effect of the Addition of Calcium on the Coagulation of Embryonic Blood.

Attention was next directed to the subject of calcium. Table V presents the results of a series of experiments showing the effect of the addition of two drops of 0.5 per cent calcium chloride to embryonic blood. Here again a reduction of over 50 per cent was obtained. While this was not so pronounced as with platelet material, the results are more uniform and show that the addition of calcium has a decided effect on the coagulation process. These experiments seem

TABLE V.

Effect of the Addition of Calcium on the Coagulation Time of Embryonic Blood.

Size of embryo.	Volume of blood.	Coagulation time.		Size of embryo.	Volume of blood.	Coagulation time.	
		Normal control.	Blood plus 2 drops of 0.5 % CaCl ₂ .			Normal control.	Blood plus 2 drops of 0.5 % CaCl ₂ .
mm.	cc.	min.	min.	mm.	cc.	min.	min.
100	0.5	30	13	180	0.5	20	11
120	0.5	28	10	190	0.5	23	12
130	0.5	30	13	200	0.5	22	11
135	0.5	30	11	210	0.5	21	9
140	0.5	21	11	220	0.5	24	9
150	0.5	22	8	240	0.5	21	12
160	0.5	20	8	250	0.5	21	8
170	0.5	21	9	270	0.5	20	9
Average.....						23.4	10.3

to show a deficiency of calcium in the embryonic blood, which would seriously interfere with coagulative processes. This conclusion, however, is contradicted by the results obtained in the platelet experiments, which indicate that there must have been a sufficient amount of calcium ions available for coagulation.

From these results the platelet experiments alone would seem to indicate a deficiency on the part of prothrombin rather than calcium, whereas in the calcium experiments the situation appears reversed, and a deficiency in calcium rather than prothrombin is indicated.

Reaction of Embryonic Blood to Tissue Extract.

It will be observed that the platelets are used in the form of a suspension. The question arises whether the effect of the suspension on coagulation may not have been due to elements other than the prothrombin content, for it is to be recognized that in addition to prothrombin, blood platelets contain a second substance, thromboplastin (Bayne-Jones (10)), corresponding in its reaction with the phosphatide designated as cephalin (Howell (11)⁵).

In the preparation of the platelet material used in the present experiments the technique was such that the thromboplastin was still retained in the suspension, and it is therefore possible that this sub-

TABLE VI.

Effect of the Addition of Tissue Extracts on the Coagulation Time of Embryonic Blood.

Size of embryo.	Volume of blood.	Coagulation time.		
		Normal control.	Blood plus 2 drops of heart extract.	Blood plus 2 drops of cord extract.
<i>mm.</i>	<i>cc.</i>	<i>min.</i>	<i>min.</i>	<i>min.</i>
100	0.5	20	4	4
140	0.5	19	4	4
170	0.5	18	3	3.5
200	0.5	13	3	4
230	0.5	18	4	4
250	0.5	15	2	3
Average.....		17.2	3.3	3.7

stance constitutes an important factor in the preceding results. To determine whether this was the case, experiments were made testing the effect of tissue extract on coagulation, since it has been shown that this active substance is present in such extract (Howell (11)⁵).

The extracts were made of the heart and umbilical cord of pig embryos about 200 mm. in size. These tissues were rinsed in water to remove any excess of blood and extracts made in saline solution and water by grinding in a mortar with sand. The mixture was then transferred to a 15 cc. centrifuge tube, centrifuged for 5 minutes, and the supernatant fluid removed. This fluid, or extract, was then used in a series of experiments with the results shown in Table VI.

⁵ Howell (11), p. 290.

These results clearly demonstrate that tissue extracts have a decided effect upon the coagulation time. Not only is the time greatly reduced, but it is brought down to the normal time for adult blood; *viz.*, 3 to 4 minutes. It appears obvious, therefore, that in spite of the fact that the embryonic blood normally shows a long coagulation time, nevertheless not only does it contain all the constituent elements essential to coagulation, but these elements will also react under proper conditions in a time equivalent to that of the adult blood.

The results were further verified by blood which had come in contact with cut tissue surfaces by being allowed to run over the cut surfaces of the heart and cord and was found to coagulate in 3 to 4 minutes. It should be stated that in all the experiments with tissue extract not only was the coagulation time thus reduced but the clot was of a much firmer character and permitted complete inversion of the test-tube.

From the standpoint of the results with tissue extract alone it would appear that the delayed coagulation time in embryonic blood must be due to some inhibitory factor. From our knowledge of tissue extracts (Howell (11)⁶) it might be assumed that this factor is solely in the nature of an antithrombin or antiprothrombin. The results obtained with calcium, the addition of which also brings about a reduction in coagulation time, are theoretically inconsistent with the preceding assumption, since this involves the neutralization of antithrombin or antiprothrombin by calcium. These facts indicate that in attempting to determine the nature of the inhibitory factor or factors in embryonic blood, consideration must be given to their possible interaction with calcium as well as with prothrombin.

Calcium Content of Embryonic and Adult Blood.

A chemical analysis of adult and embryonic blood was undertaken to ascertain the calcium content of embryonic blood.⁷ The results show that instead of a deficiency there is a pronounced excess of cal-

⁶ Howell (11), p. 292.

⁷ This work was done by Dr. P. G. Albrecht, of the Department of Physiological Chemistry, who kindly furnished us with data from his unpublished results (Table VII).

cium present in embryonic blood as compared with adult blood (Table VII).

Since on the one hand the results of the preceding experiments upon the effect of the addition of calcium indicate a deficiency in this element, while on the other hand the present data show that calcium is quantitatively present in excess, the suggestion arises that the calcium in embryonic blood is present in some combined form which renders the blood deficient in the free calcium ions essential to coagulation.

TABLE VII.

*Determination of Calcium for Embryonic and Adult Blood.**

Quantity.	Amount of calcium.		Ratio of embryo to adult.
	Embryo (200 mm.).	Adult.	
	mg.	mg.	
Total blood 100 gm.	9.5	6.74	7 : 5
Plasma 100 cc.	11.73	8.51	7 : 5

* McCrudden's method as modified by Halverson and Bergeim was used (Halverson, J. O., and Bergeim, O., *J. Biol. Chem.*, 1917, xxxii, 159).

Effect of the Addition of Barium and Magnesium on the Coagulation of Embryonic Blood.

Since the calcium is, therefore, evidently present in embryonic blood in some combined form, it becomes of interest to ascertain whether the addition of other salts, such as barium and magnesium, might liberate some of this calcium and bring about a change in coagulation time. With this end in view experiments were made with the chlorides of barium and magnesium in 0.5 per cent strength (Table VIII).

As a control to determine that these results were due to a liberation of calcium and not to an interaction with some other elements, such as prothrombin for example, the same experiments were repeated on oxalated embryonic blood as shown in Table IX.

The results are significant. Like calcium, barium and magnesium, although somewhat slower in reaction and giving a clot of somewhat smaller volume and looser texture, bring about a reduction in coagulation time of non-oxalated embryonic blood, as shown in Table VIII. These elements, in contrast to calcium, do not react with the oxalated

embryonic blood. On the basis of the specificity of calcium these results apparently permit only one conclusion; namely, that the action of barium and magnesium is due to a liberation of an amount of cal-

TABLE VIII.

Effect of the Addition of Calcium, Barium, and Magnesium on the Coagulation Time of Embryonic Blood.

Size of embryo.	Volume of blood.	Coagulation time.			
		Normal control.	Blood plus 2 drops of 0.5% CaCl_2 .	Blood plus 2 drops of 0.5% BaCl_2 .	Blood plus 2 drops of 0.5% MgCl_2 .
<i>mm.</i>	<i>cc.</i>	<i>min.</i>	<i>min.</i>	<i>min.</i>	<i>min.</i>
135	0.5	25	11	14	18
180	0.5	15	6	8	8
230	0.5	18	6	7	7
250	0.5	21	8	8	10
270	0.5	15	9	10	11
Average.....		19	8	9.8	10.8

TABLE IX.

Effect of the Addition of Calcium, Barium, and Magnesium on the Coagulation Time of Oxalated Embryonic Blood.

Size of embryo.	Volume of blood.	Coagulation time.			
		Normal control.	Blood plus 3 drops of 0.5% CaCl_2 .	Blood plus 3 drops of 0.5% BaCl_2 .	Blood plus 3 drops of 0.5% MgCl_2 .
<i>mm.</i>	<i>cc.</i>	<i>min.</i>	<i>min.</i>	<i>min.</i>	<i>min.</i>
170	0.5	No clot.	6	No clot.*	No clot.†
270	0.5	" "	7‡	" " §	" " §

* Two more drops of barium added after $\frac{1}{2}$ hour; no clot formed. Three drops of calcium added after 1 hour; clot formed in 6 minutes.

† Two more drops of magnesium added after $\frac{1}{2}$ hour; no clot formed. Three drops of calcium added after 1 hour; clot formed in 7 minutes.

‡ Two drops of calcium used instead of three.

§ Two drops of calcium added after 25 minutes; clot formed in 5 minutes.

cium sufficient to permit the interaction essential for coagulation, thus furnishing confirmatory evidence for the conclusion that the calcium is present in some combined form.

Certain experiments (Table X) have shown that embryonic blood treated with an amount of oxalate sufficient to prevent coagulation can be made to coagulate upon the addition of tissue extract. On the other hand, in similar experiments with adult blood, in no instances where oxalation was sufficient to prevent completely coagulation could coagulation be initiated by the addition of tissue extract.

TABLE X.

Effect of the Addition of Tissue Extract on the Coagulation Time of Oxalated Blood.

Source of blood.	Volume of blood.	Sodium oxalate.		Tissue extract.	Coagulation time.
		<i>per cent</i>	<i>glt.</i>	<i>glt.</i>	
170 mm. embryo.	0.5				19 min.
	0.5			2	2.5 "
	0.5	0.5	1		No clot in 1 hr.
	0.5	0.5	1	3	9 min.
	0.5	1	1		No clot in 1 hr.
	0.5	1	1	3	" " " 1 "
	0.5	1	2		" " " 1 "
	0.5	1	2	3	" " " 1 "
200 mm. embryo.	0.5				13 min.
	0.5			3	2 "
	0.5	0.5	1		No clot in 1 hr.
	0.5	0.5	1	3	5 min.
	0.5	1	1		No clot in 1 hr.
	0.5	1	1	3	" " " 1 "
Adult.	0.5	0.5	1		3 min.
	0.5	0.5	1	3	3 "
	0.5	1	1		3 "
	0.5	1	1	3	3 "
	0.5	1	2		No clot.
	0.5	1	2	3	" "

Fibrin and Fibrinogen.

Regarding fibrinogen, the third of the factors under consideration as essential to coagulation, the following observations were made. With the other two factors it has been possible to show that in a comparison of embryonic and adult blood of the pig, the calcium and platelet content in the blood of 100 to 270 mm. embryos is more or less

comparable with that of the adult. With fibrin certain differences were observed in the course of the preceding experiments. In the coagulation of embryonic blood it was observed that the clot remaining after complete retraction was somewhat larger in tissue extract experiments than in calcium experiments. In both instances, however, the retracted clots were larger than the corresponding resultant clots for normal embryonic blood.

To compare the relative quantities of fibrin formed in embryo and adult, 0.5 cc. quantities of adult and embryonic blood were defibrinated by stirring with clean glass rods drawn out to narrow points.

TABLE XI.

Weight of Fibrin Obtained in the Coagulation of Embryonic and Adult Blood.

Source of blood.	Volume of blood.	Substance added.	Weight of fibrin.
	<i>cc.</i>	<i>glt.</i>	<i>mg.</i>
200 mm. embryo.	0.5		0.1
	0.5	Calcium, 3	0.3
	0.5	Platelets, 3	0.2
	0.5	Tissue extract, 3	0.5
190 mm. embryo.	0.5		0.1
	0.5	Calcium, 3	0.3
	0.5	Platelets, 3	0.1
	0.5	Tissue extract, 3	0.5
Adult.	0.5		4.2
	1.0		8.5

Tests were made with adult blood, normal embryonic blood, and normal embryonic blood to which calcium, platelets, and tissue extract had been added. The fibrin collected on the glass rods was freed from blood by washing in distilled water and kept in 0.5 per cent formaldehyde until weighed. It was then carefully transferred to weighing tubes, dried over night at 55°C., and weighed. Table XI shows the relative quantities of fibrin obtained from embryonic and adult blood.

These results appear to demonstrate clearly a pronounced difference between embryo and adult. In the coagulation of normal embryonic blood only a small amount of fibrin is formed, whereas more fibrin

is formed upon the addition of calcium, and the maximum amount is obtained in the presence of tissue extract. The variation with platelet material is probably due to corresponding variations in the strength of the suspension used. The maximum amount of fibrin formed in embryonic blood is about 12 per cent of that obtained from an equal volume of adult blood. If the quantity of fibrin can be taken as a fairly reliable index of the relative amount of fibrinogen present, it appears that, in contrast to the comparative calcium and platelet content, the fibrinogen content of embryonic blood is far below that of adult blood. This difference in fibrin and fibrinogen content is evidently not primarily responsible for the greater coagulation time of embryonic blood, for in the presence of tissue extract, in spite of the smaller amount of fibrinogen, the coagulation time becomes equivalent to that of the adult (Tables II and VI).⁸

Bile.

The delay in coagulation of embryonic blood is primarily a result of some condition pertaining to calcium. Furthermore, it has also been shown that this is due not to a deficiency in the calcium content, but to some combination of the calcium which renders it unavailable for coagulation.

A consideration of the various types of pathological hemorrhage in man shows that in jaundice or icterus conditions are encountered which are especially significant with reference to the present subject. In jaundice there is a marked delay in the coagulation time of the blood. In the history of the subject this delay has been assigned to

⁸ In a microscopic study of the formation of fibrin an observation was made on the behavior of erythrocytes which seems worthy of note. Gütig in a study of adult pig blood states that the erythrocytes never show rouleau formation (Gütig, K., *Arch. mikr. Anat.*, 1907, lxx, 635). In contrast to this, in our own experiments with vaseline-sealed preparations of adult blood, definite and characteristic rouleau formations were observed. It appears much more difficult to demonstrate rouleau formation in the pig than in man, but rouleaux may nevertheless be obtained in great abundance in preparations of pig blood, if care is taken not to use a drop so large as to obliterate the rouleaux or so small as to result in too great pressure of the cover-glass upon the corpuscles. In embryonic blood rouleau formation was not observed even in the oldest embryos.

various causes, but in the more recent literature, as demonstrated by Lee and Vincent (12) and stated by Wells (13), there is a convergence of opinion toward the conclusion that "in icterus a notable tendency to hemorrhage seems to depend upon the binding effect of the calcium of the blood by the bile pigments."⁹

This conclusion becomes highly suggestive with reference to embryonic blood, for it has recently been shown by Bang (14) that in the new-born infant a notable amount of bile pigment is constantly present in blood obtained from the umbilical cord. This pigment shows a constant increase during the first few days, and, if marked, the transition is readily made to the condition designated clinically as icterus neonatorum. Ylppö (15) in his earlier article on new-born and premature infants obtained similar results, but with the exception of the horse, comparable conditions were not found in other mammals. In the wolf, deer, leopard, yak, dog, and pig, in contrast to horse and man, only small quantities of bile pigment, if any, were found in blood of the new-born animal at birth. He concludes that toward the end of the intrauterine life of the horse and man there is a quantity of bile constantly present in the circulating blood, and suggests that the same may be true in the intrauterine life of mammals in general, but in the latter case the excess of bile in the circulation has largely disappeared at the time of birth; or in other words, that with respect to the functional activity of the liver and excretion of bile, the young of the horse and man as compared with other mammals may be regarded as prematurely born.

These results with reference to newly born animals suggest the possibility of the presence of bile in the blood of the pig embryos used in the present experiments and that this bile may be a factor of primary importance affecting the process of coagulation. To determine this point a qualitative examination was made of serum obtained from the blood of 100 to 270 mm. embryos. Tests were made in accordance with Huppert's technique in which the bile pigments are precipitated with milk of lime, freed from the precipitate by boiling in strong acid-alcohol, and their presence demonstrated by the appearance of a typical color reaction. 25 cc. of serum were obtained from

⁹ Wells (13), p. 321.

several embryos and the precipitate formed with the milk of lime was treated with 10 to 15 cc. of the acid-alcohol, thus securing the pigment in more concentrated form for the final color test. The results furnished conclusive evidence of the presence of bile in the blood of the pig embryos.

As already indicated, it has been shown that in icterus (Lee and Vincent (12)) the presence of bile may interfere with the participation of calcium in coagulation, and this suggests that the same may be true of embryonic blood. Since the coagulation time for embryonic blood could be greatly reduced by the addition of calcium, and even brought down to the coagulation time obtained for the adult by the addition of tissue extract, as demonstrated in the preceding experiments, it became important to ascertain whether similar experimental conditions could be produced in normal adult blood through the presence of bile.

To this end the following experiments were made in which given concentrations of bile obtained from gall bladders of adult pigs were added to definite quantities of adult pig blood. It was found that the amount and concentration of bile added to the blood is of primary importance in obtaining a given effect on the coagulation time. In order to approximate embryonic conditions, the addition of bile should be such as to bring about a coagulation of the adult blood in about 20 to 30 minutes. To ascertain the requisite amount of bile for this purpose, tests were made with various concentrations (Table XII). The bile for this purpose was removed from the gall bladder, filtered, and then diluted with distilled water to give the percentages of concentration indicated.

On the basis of these experiments, it was determined that three drops of 20 to 25 per cent solution of bile constitute the optimum amount necessary to give a coagulation time of about 20 to 30 minutes for adult blood. The blood when thus treated contains 8 to 10 per cent bile in the final mixture. After the optimum amount of bile necessary for the present purpose had been determined, experiments were made to test the coagulation reaction of adult blood to which bile had been added (Table XIII).

Table XIV gives the weight of fibrin obtained from adult blood treated with bile.

TABLE XII.

Effect of Different Concentrations of Bile on the Coagulation Time of Adult Blood.

Volume of blood.	Bile.		Coagulation time.	Volume of blood.	Bile.		Coagulation time.
	Concentration.	Amount added.			Concentration.	Amount added.	
cc.	per cent	gtl.	min.	cc.	per cent	gtl.	min.
0.5	1	2	3	0.5	20	3	27
0.5	3	2	3	0.5	25	1	14
0.5	5	2	3	0.5	25	1	10
0.5	7	2	3	0.5	25	2	22
0.5	10	2	3	0.5	25	3	30
0.5	20	1	3	0.5	25	3	27
0.5	20	2	6	0.5	25	3	25
0.5	20	2	7	0.5	50	1	120
0.5	20	3	30	0.5	100	1	No clot.*
0.5	20	3	20	0.5	100	2	" " *

* Only loose strands of fibrin found.

TABLE XIII.

Coagulation Time of Adult Blood in the Presence of Bile.

Volume of blood.	Coagulation time.					
	Blood plus 3 drops of bile.	Normal control.	Blood plus 3 drops of 0.5% CaCl ₂ .	Blood plus 3 drops of 0.5% BaCl ₂ .	Blood plus 3 drops of 0.5% MgCl ₂ .	Blood plus 3 drops of tissue extract.*
cc.	per cent	min.	min.	min.	min.	min.
0.5	20	27	15			4
0.5	20	20	8			3
0.5	25	25	8	10	10	2
0.5	25	27	12	13	15	3
Average.		24.75	10.75	11.5	12.5	3

* Extract was made from embryonic heart.

TABLE XIV.

Weight of Fibrin Obtained in the Coagulation of Adult Blood in the Presence of Bile.

Volume of blood.	Amount of 25% bile added.	Other substances added.	Weight of fibrin.
cc.	gtl.	gtl.	mg.
0.5			4.2
0.5	3		1.2
0.5	3	Calcium 0.5%, 4	5.4
0.5	3	Tissue extract, 4	5.0

These results appear significant. The coagulation time of the adult blood when delayed to about 25 minutes by the addition of bile can, by the addition of calcium, barium, and magnesium, be reduced to an average of 10.75, 11.5, and 12.5 minutes, respectively, and upon the addition of tissue extract it can be brought back to the normal time of 3 minutes. Again, whereas in the presence of bile the amount of fibrin formed is reduced to about 30 per cent of the normal, the addition of tissue extract brings the amount back to normal and indeed in the present experiment slightly in excess. These results accord to a remarkable degree with the results previously obtained for the embryo (Tables V, VI, VIII, and XI) and demonstrate that by the mere addition of bile conditions can be produced in the adult blood which appear essentially identical with those of the embryonic blood.

Since in the course of the preceding analysis of the factors involved in coagulation no significant differences could be demonstrated between the blood of the embryo and that of the adult, other than the presence of bile in the former, the data justify the conclusion that it is the bile content found in the circulation of 100 to 270 mm. pig embryos that constitutes the primary factor in the greater coagulation time of their blood.

DISCUSSION.

When, in the earlier phases of this study, it was found that the greater coagulation time of embryonic blood could not be due to a quantitative deficiency in blood platelets as compared with adult blood, the suggestion arose that possibly the condition might be comparable with that of hemophilia, for it seems to be well established that in the latter condition also the platelet content numerically is essentially equivalent to that of normal blood (Lee and Minot (7)³). Subsequent data, however, have shown that such a comparison cannot be made. On the contrary, instead of the condition in the embryonic blood being comparable with hemophilia, it has been found to be more nearly that of icterus in which the bile constitutes the primary factor in the abnormal coagulation time.

Since it appears that the inhibitory action of bile is due in a large degree to the combination of the pigment with calcium (Wells (13)), the question arises as to how the process of coagulation is initiated in

normal embryonic blood by the addition of tissue extract. In the calcium experiments the results are due primarily to the introduction of calcium ions in excess of the amount which enters into chemical combination with the bile present. With tissue extract apparently the free calcium ions requisite for the initiation of coagulation must be liberated through some interaction with the constituents of bile. Such an interaction seems clearly demonstrated in the preceding experiments with adult blood to which bile had been added (Table XIII). This is in contrast to the results of Lee and Vincent (12) who, in their study of obstructive jaundice, state that they "attempted to determine whether the action of bile could be neutralized by cytozyme or serozyme, but no results were obtained."¹⁰

This suggests that the normal coagulation of embryonic blood, as far as bile is concerned, involves a process comparable with that observed after the addition of tissue extract or cephalin, but on a smaller scale. In the embryonic blood *in vitro*, through the disintegration of cellular elements, a certain amount of tissue substance (cephalin (?)) is slowly set free in the plasma. This tissue substance neutralizes the bile and ultimately liberates a sufficient amount of calcium to bring about coagulation.

Of interest in this connection is the fact that the first formation of fibrin takes place almost invariably at the side of the tube (page 180). This fibrin is situated at a level between the red cells and supernatant plasma, probably because of the disintegration of the white cells and platelets which tend to accumulate at this level, an observation confirmatory of that made by Schmidt (16) "that clotting begins always in the white layer." These cellular elements coming in contact with the sides of the tube evidently liberate some substance presumably comparable with cephalin which in turn initiates the formation of fibrin at this location.

The fact that there is a pronounced difference in the quantity of fibrinogen in the blood of the embryo as compared with that of the adult, and that under proper conditions the coagulation time becomes the same for both embryo and adult, demonstrates that the quantity of fibrinogen present in the blood does not play an important part in

¹⁰ Lee and Vincent (12), p. 63.

determining the coagulation time of blood, a conclusion confirmatory of Whipple's observation (17)¹¹ in certain cases of purpura and hepatic cirrhosis, but in marked contrast to Ottenberg's statement (18) that "in hemorrhagic diseases of the newborn and in chloroform poisoning the prolonged coagulation time is due to a deficiency of fibrinogen."¹²

While the amount of fibrinogen is, therefore, of minor importance as far as coagulation time is concerned, it is to be noted that the actual quantity of fibrin formed in the embryonic blood was greater upon the addition of tissue extract than in the experiments with calcium. In the recent study of the effect of bile on the clotting time of blood, Haessler and Stebbins (19) conclude that bile, through its bile salts, interferes with the conversion of fibrinogen into fibrin. It is consequently possible that the greater amount of fibrin obtained with calcium may be due to its interaction primarily with bile pigments, whereas the action of tissue extract involves not merely the bile pigments but also the bile salts, thus accounting for the increased amount of fibrin. On the other hand, it must be observed that in the experiments with adult blood and bile (Table XIV), upon the addition of calcium an amount of fibrin was obtained essentially equivalent to that obtained upon the addition of tissue extract.

SUMMARY.

1. In pig embryos of 100 to 270 mm. the average coagulation time of the blood was found to be about 23 minutes. This represents a coagulation time six to eight times greater than that obtained for the adult.

2. The first evidence of coagulation in the blood of these embryos consisted in the appearance of small masses of fibrin deposited almost invariably at the side of the test-tube. The ensuing coagulum was, as a rule, in the nature of a sliding clot, never attaining any marked degree of density or firmness.

3. In an analysis of the possible factors involved in this greater coagulation time of embryonic blood, it was found that numerically the blood platelets varied from 415,000 to 800,000 per c.mm., a content not differing in any significant degree from that of the adult, in

¹¹ Whipple (17), p. 390.

¹² Ottenberg (18), p. 308.

which the average was found to be about 588,000 with a variation from 544,000 to 932,000.

4. The addition of platelet material obtained from adult pig blood reduced the coagulation time for embryonic blood to an average of 8.4 minutes, a decrease of 75 per cent.

5. The addition of two drops of 0.5 per cent calcium chloride reduced the coagulation time for embryonic blood to an average of 10.3 minutes, a reduction of over 50 per cent.

6. The addition of tissue extract to embryonic blood reduced the coagulation time to an average of 3.7 minutes, a time essentially equivalent to that obtained for adult blood. The clot was of a much firmer character than that obtained either in the normal coagulation or in the calcium experiments.

7. Chemical analysis demonstrated a calcium content in embryonic blood in excess of that of the adult, in the proportion of 7 : 5.

8. In view of the results indicated above, it became evident that the calcium in embryonic blood must be present in some combined form. This conclusion is supported by experiments in which barium and magnesium brought about a reduction in coagulation time in non-oxalated embryonic blood, but did not have this effect when added to oxalated blood, indicating in the former case, on the basis of the specificity of calcium, a liberation of free calcium ions. With oxalated blood, it was also found that under certain conditions coagulation could be brought about by the addition of tissue extract.

9. That the fibrinogen content plays no important part in coagulation time is shown by the fact that whereas the maximum amount of fibrin obtained by defibrinating embryonic blood was about 12 per cent of that obtained from the adult, nevertheless upon the addition of tissue extract the coagulation time of embryonic blood becomes equivalent to that of the adult.

10. The presence of bile was demonstrated in the circulating blood of these embryos. Since it was further demonstrated that by the addition of bile to adult blood conditions could be produced essentially identical with those of embryonic blood, and since in the preceding analysis of the factors involved in coagulation no significant differences could be demonstrated between the blood of the embryos and that of the adult other than the presence of bile in the former, the

data justify the conclusion that the bile content in the circulation of 100 to 270 mm. pig embryos constitutes the primary factor accounting for the greater coagulation time in the blood of these embryos. A condition in embryonic blood in some respects comparable with that of icterus is also indicated.

11. In the calcium experiments the results are apparently due primarily to the introduction of calcium ions in excess of the amount which enters into chemical combination with the bile present. With tissue extract it appears that the free calcium ions essential for the initiation of coagulation must be liberated through some interaction with the constituents of bile.

12. The results of this study suggest that the normal coagulation of embryonic blood, as far as bile is concerned, involves a process comparable with that obtained after the addition of tissue extract or cephalin, but on a smaller scale. In the embryonic blood *in vitro*, through the gradual disintegration of cellular elements, a certain amount of tissue substance (cephalin(?)) is slowly set free in the plasma, neutralizing the bile and ultimately liberating a sufficient amount of calcium to bring about coagulation.

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CRESCENTIC BODIES IN ÆSTIVO-AUTUMNAL MALARIA; THEIR MIGRATION AND ATTACHMENT TO THE SURFACE OF THE RED CORPUSCLE.*

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PLATE 13 AND 14.

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Æstivo-Autumnal Parasites.

Since 1879, when malarial parasites were first described, the consensus of opinion has been that the parasites are intracellular, and that each parasite, when young, enters a red corpuscle, staying there until it dies or segmentation takes place, the destruction of the corpuscle corresponding with the segmentation of the parasite. But this is not the case. All malarial parasites are extracellular, that is they are attached to the external surface of the infected corpuscle, and each parasite destroys several red corpuscles. One may trace the destruction of corpuscle after corpuscle by the migrating parasites of æstivo-autumnal infections.

The parasites usually present in the blood of æstivo-autumnal infections are the ring-forms and crescentic bodies. When round bodies are present, they are usually developmental forms of the crescent. The crescent may present an ovoid appearance (1) when bent on itself and viewed from the convex side and (2) when contracted. When the parasite is contracted, the width of the body is increased.

Crescentic Bodies.

The shape of the crescent is similar to that of a caterpillar; the extremities are generally blunt, but one or both may be pointed. That the body of the parasite is wider than it is thick is illustrated by the appearance of one when bent on itself (Figs. 6, 37, and 49).

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Attachment of the Crescent to the Red Corpuscle.—The parasite is extracellular and wraps itself around the corpuscle as a worm wraps itself around a berry (Figs. 1 to 10 and 36). The attachment of these bodies to the external surface of the red corpuscle is demonstrated by the following facts. (a) When attached to a corpuscle and seen in profile, the poles of the parasite may be observed extending beyond the periphery of the infected corpuscle in many instances (Figs. 1 to 3, 5 to 8, and 10). (b) When viewed from above, one occasionally sees the poles of the attached crescent extending beyond the periphery of the corpuscle (Figs. 9, 12, 26, 30, 32 to 34, and 38 to 40), and in these instances it is often possible to trace the outline of the corpuscle crossing the body of the parasite (Figs. 32, 34, 38, and 39). In rare instances the body proper of the crescent may be seen protruding beyond the periphery of the infected corpuscle (Fig. 48; the dotted line shows where the corpuscle crosses the parasite). (c) Occasionally the corpuscle may be seen situated between the body proper and the clubbed end of a crescent bent on itself (Fig. 37).

Ordinarily the corpuscle appears only on one side of the crescent, especially after the corpuscle has been decolorized; but occasionally one may see the corpuscle projecting on either side of the crescent. In these instances the parasites show but little curving and one views them from above (Figs. 13 to 16, 18, 19, 26 to 28, 31, 33, 35, 39, and 40). In the majority the hemoglobin of the corpuscles is more or less intact. Under such circumstances, where the parasite is attached to its full extent, the picture is what one would expect, as the healthy corpuscle is more elastic than the parasite, and spreads out in all directions when pressure is exerted.

Hemoglobin Mounds.

The mounds of hemoglobin substance which the parasite encircles with its cytoplasm for the purpose of attachment and assimilation are easily demonstrated when seen at the periphery of the parasite (at o in Figs. 1 to 3, 5, 6, 29, 32, 35, 47, and 49). These mounds are usually decolorized before the corpuscle itself, but occasionally they may be seen not yet decolorized, in connection with parasites attached to corpuscles showing varying stages of dehemoglobinization, and to corpuscular skeletons (Figs. 41, 43, and 46, at o).

Migration of the Crescentic Bodies.

Crescentic bodies go through migratory stages similar to those of other malarial parasites, and it is possible to find all the stages in one film, especially if the infection is a heavy one and quinine has not been given.

Evidence of Migration.—That the crescentic bodies destroy more than one red corpuscle is strongly suggested by the following facts. (a) Heavily pigmented parasites may be found attached to newly invaded red corpuscles. By newly invaded, I mean instances where the hemoglobin appears to be as yet unaltered by the action of the attached parasites (Figs. 1 to 5, 8 to 10, 12, 14 to 20, 22 to 28, and 56). The pigmentation of these parasites is evidence of previous attachments. (b) In the same film pigmented parasites are observed on red corpuscles showing varying degrees of dehemoglobinization (Figs. 6, 7, 11, 21, 29 to 40, 47 to 49, and 53). (c) Pigmented parasites occur on corpuscular skeletons or remnants of red corpuscles which have been altered by the action of the attached parasites (Figs. 41 to 46). These skeletons may also be seen free from parasites. (d) In the same film pigmented parasites are found free from red corpuscles (Figs. 50 to 52, 54, and 55). The pigmentation of these parasites is evidence of previous attachments.

Occasionally a crescent is seen in the act of attaching itself to a fresh red corpuscle before it has conformed itself to the curved surface of the corpuscle (Fig. 56; a careful examination of this figure will show clearly the attachment of the parasite to the corpuscle).

I have never observed the migratory stages in connection with the crescents after the administration of quinine. Possibly the continued use of quinine may inhibit the migration of the crescents without causing their immediate destruction, as they may be present in the blood for some time after quinine has been used without any appreciable anemia resulting. Migration of the crescentic bodies is less frequently observed in the blood than is migration of the young forms of the æstivo-autumnal parasite. This finding might be explained by the fact that the infection is usually recognized before the appearance of the crescents and vigorous treatment instituted. But the large amount of pigment which most of these bodies contain

would indicate that they may do considerable damage before their destructive action is restrained.

A heavily pigmented, full grown parasite attached to a red corpuscle the hemoglobin of which is intact, or nearly so, should suggest that the parasite must have obtained that pigment from another source, and that source was undoubtedly another red corpuscle. It seems to me that this is convincing evidence of parasitic migration.

There is considerable evidence of the migration of the crescentic bodies in the observations of several writers. Free crescents and heavily pigmented crescents attached to healthy appearing red corpuscles have been described and frequently illustrated. Osler¹ pictures free pigmented crescents. Laveran² pictures free pigmented crescents and a pigmented parasite attached to a healthy appearing red corpuscle. Mannaberg³ pictures free crescents and pigmented crescents attached to healthy appearing red corpuscles. Canalis⁴ states that the crescents decolorize the red corpuscles and finally become free in the blood, and he illustrates⁵ free crescents and pigmented crescents attached to healthy appearing red corpuscles. Thayer and Hewetson,⁶ in describing crescents, state that in some instances all trace of the corpuscle may be absent, and Thayer⁷ gives figures showing a pigmented crescent on a corpuscle that has not been decolorized, and two free crescents. Manson⁸ pictures a pigmented crescent attached to a healthy appearing red corpuscle. Marchiafava and Bignami⁹ show a pigmented cres-

¹ Osler, W., quoted from Laveran, A., *Paludism*, translation by Martin, J. W., London, 1893, 41, Fig. 7, F and H.

² Laveran, A., *Paludism*, translation by Martin, J. W., London, 1893, 18, Fig. 3.

³ Mannaberg, J., *The malarial parasites. A description based upon observations made by the author and by other observers*, translation by Felkin, R. W., London, 1894, Plate 2, Figs. 51 and 52, Plate 4, Figs. 35 to 42, 53, and 54.

⁴ Canalis, P., quoted from Thayer, W. S., and Hewetson, J., *The malarial fevers of Baltimore, Johns Hopkins Hosp. Rep.*, 1895, v, 24.

⁵ Canalis, P., *Studi sulla Infexione malarica. Sulla varietà parassitaria delle forme semilunari di Laveran e sulle fibbri malariche che da esse dipendono*, *Arch. sc. med.*, 1890, xiv, 75, Plate 3 C, Figs. 4, 5, and 12, 3 B, Figs. 7 to 9.

⁶ Thayer, W., and Hewetson, J., *The malarial fevers of Baltimore, Johns Hopkins Hosp. Rep.*, 1895, v, 93.

⁷ Thayer, W. S., *Lectures on the malarial fevers*, New York, 1897, Plate 3, Figs. 29, 33, and 41.

⁸ Manson, P., *Tropical diseases: a manual of the diseases of warm climates*, London, Paris, New York, and Melbourne, 2nd edition, 1900, 14, Fig. 9.

⁹ Marchiafava, E., and Bignami, A., *Malaria*, in Stedman, T. L., *Twentieth century practice*, New York, 1900, xix, Plate 2, Figs. 54, 61, 64, and 65.

cent attached to an apparently healthy red corpuscle, as well as free crescents. Celli¹⁰ pictures a pigmented crescent attached to a healthy appearing red corpuscle and a free crescent. Brumpt¹¹ pictures a free crescent and Thompson¹² heavily pigmented crescents attached to red corpuscles whose hemoglobin appears to be intact, as well as crescents free from red corpuscles.

EXPLANATION OF PLATES.

PLATE 13.

Magnification, $\times 1,684$.

FIGS. 1 to 5. Heavily pigmented crescents attached to the periphery of healthy appearing red corpuscles, the hemoglobin of which appears to be intact. Hemoglobin mounds encircled by the cytoplasm of the parasites may be seen at o.

FIG. 6. A heavily pigmented crescent attached to a slightly decolorized red corpuscle. Hemoglobin mounds may be seen at o and an attaching filament from the cytoplasm of the parasite may be seen at x. This filament extends beyond the periphery of the infected corpuscle.

FIG. 7. A heavily pigmented crescent attached to a slightly decolorized red corpuscle. The poles of the crescent may be seen extending outside the periphery of the corpuscle.

FIG. 8. A heavily pigmented crescent attached to a healthy appearing red corpuscle, the hemoglobin of which appears to be intact. An attaching filament extending beyond the infected corpuscle is seen at x.

FIG. 9. A heavily pigmented parasite attached to a healthy appearing red corpuscle. The clubbed ends of the crescent may be seen bent over the edge of the infected red corpuscle.

FIG. 10. A heavily pigmented parasite attached to a healthy appearing red corpuscle. The clubbed end at the lower part of the picture is seen extending beyond the periphery of the infected corpuscle.

FIGS. 11 and 12. Heavily pigmented parasites attached to fairly healthy appearing red corpuscles.

FIG. 13. Crescentic body attached to a red corpuscle showing Schüffner's granules.

FIGS. 14 to 21. Heavily pigmented parasites attached to healthy appearing red corpuscles.

FIG. 22. A heavily pigmented crescent attached to a healthy appearing red corpuscle. A flagellum taking the nuclear stain may be seen at x.

¹⁰ Celli, A., *Malaria*, New York, 1901, 45, Figs. N and R.

¹¹ Brumpt, É., *Paludisme, Précis de parasitologie*, Paris, 2nd edition, 1913, Plate 1, Fig. 14.

¹² Thompson, D., *The origin and development of gametes (crescents) in malignant tertian malaria: some observations on flagellation, etc.*, *Ann. Trop. Med. and Parasitol.*, 1914-15, viii, 85, Plate 5, Figs. 12 to 14 and 28 to 32.

FIGS. 23 to 29. Heavily pigmented crescents attached to red corpuscles apparently containing a normal amount of hemoglobin. Fig. 29 shows hemoglobin mounds at o.

FIG. 30. A heavily pigmented crescent attached to a corpuscle partly dehemoglobinized. What is probably an attaching filament, arising from the cytoplasm of the parasite, may be seen extending from x to x.

FIGS. 31 to 35. Heavily pigmented crescents attached to red corpuscles in varying stages of dehemoglobinization. Hemoglobin mounds may be seen at o. In Figs. 32 to 34 the poles of the parasites may be seen to extend beyond the periphery of the corpuscles to which the parasites are attached. In Fig. 33 one of Maurer's rings may be seen in the infected corpuscle at x. In Fig. 35 an attaching filament arising from the cytoplasm of the parasite may be seen at x.

PLATE 14.

Magnification, $\times 1,684$.

FIG. 36. A heavily pigmented crescent wrapped around a decolorized red corpuscle. The external relation of this parasite to the corpuscle is clearly shown.

FIG. 37. A heavily pigmented crescent attached to a decolorized red corpuscle. At x the corpuscle may be seen between the body of the parasite and one of the poles. This parasite could not be within the infected corpuscle.

FIGS. 38 and 39. Heavily pigmented crescents attached to decolorized red corpuscles. The poles of the attached parasites may be seen extending beyond the periphery of the unbroken outline of the infected corpuscles. The outline of the corpuscles may be traced through the bodies of the attached parasites.

FIG. 40. A heavily pigmented crescent attached to a decolorized red corpuscle. At A the crescent is bent over the periphery of the infected corpuscle.

FIGS. 41 to 46. Heavily pigmented, healthy appearing crescents attached to corpuscular skeletons. Note the general semilunar appearance of the skeletons. Hemoglobin mounds may be seen at o. Fig. 42 shows the crescent bent on itself. Attaching filaments arising from the cytoplasm of the crescent and attached to the corpuscular skeleton may be seen at x in Fig. 46.

FIG. 47. Pigmented crescents attached to decolorized red corpuscles. Hemoglobin mounds may be seen at o.

FIG. 48. A pigmented crescent attached to a decolorized red corpuscle. The body of the attached crescent extends beyond the periphery of the decolorized corpuscle. The dotted line marks where the corpuscle crosses the parasite.

FIG. 49. A pigmented crescent attached to a decolorized red corpuscle. The crescent is bent on itself, thus giving the appearance of a double bib. A hemoglobin mound may be seen at o.

FIGS. 50 to 53. Flagellated crescents. With the exception of Fig. 53, which is attached to a decolorized corpuscle, these crescents are free from corpuscles. They are healthy appearing and the presence of pigment is evidence of previous attachments. The flagella arise from the chromatin substance of the parasites.

FIGS. 54 and 55. Free pigmented crescents.

FIG. 56. A very heavily pigmented crescent which, I believe, is in the process of attaching itself to a fresh corpuscle, as the red corpuscle shows no evidence of injury to its hemoglobin. A careful examination of this figure will show the hemoglobin of the red corpuscle extending into the substance of the crescent. The large amount of pigment which the parasite contains is evidence of previous attachments.



(Lawson: Estivo-autumnal malaria.)



(Lawson: Aestivo-autumnal malaria.)

THE HEMIC BASOPHIL.*

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PLATE 15.

(Received for publication, September 27, 1919.)

The basophilic or gamma granule of Ehrlich is generally accepted as a characteristic feature of two cell types, the histogenous or tissue basophil, or mast cell, and the basophilic granulocyte of the circulating blood, to which also the term mast cell is commonly applied. Neither of these cells is well understood. Both seem to be characterized in a biological sense by a certain inertia, a certain insensitiveness that manifests itself in a lack of response to irritants capable of arousing quick reaction on the part of cells that seem in general to be of homologous type. In this peculiarity lies, perhaps, part of the explanation for our slight knowledge of the cells in question. The basophil of the blood was, however, placed by Ehrlich among the marrow cell derivatives, since he recognized in it certain similarities to the other granule-bearing cells of the blood and bone marrow. The position thus taken has been opposed by some workers but has nevertheless gained wide acceptance, and the cell is commonly regarded as the complete homologue of the neutrophilic and eosinophilic leucocytes, from which it is believed to differ only in the basophilic staining reaction of its distinguishing granule. The origin and cellular relations as well as the possible function of the tissue mast cell are unknown. Regarded by Ehrlich as an overnourished connective tissue cell, it has been considered by other writers as a clasmatoocyte or as derived from one, and again has been looked upon as arising from the lymphocyte or plasma cell. Its possible relation to the leucocytic basophil has been the subject of special debate.

* Read before the American Association of Pathologists and Bacteriologists at Atlantic City, June 16, 1919.

It has been considered as identical with the latter, but has been more frequently regarded as a separate cell type having nothing in common with the basophil of the blood stream beyond the possession of the same or of a closely similar affinity for certain basic dyes.

Maximow (1910), in his classic studies of the connective tissue elements, concluded that in the lower vertebrates one form may pass over into the other, but in mammals he considered them as entirely separate and distinct except in as far as both are derived from an undifferentiated mesenchymal cell which he calls a lymphocyte or lymphocytoid wandering cell. However, he suggests (1906) that the two forms may be correlated functionally in some way, since the frequency of the respective types varies in different animal species in inverse ratio. Thus the rabbit has few histogenous mast cells in its tissues, but the blood carries a relatively high percentage of basophilic leucocytes, while the contrary condition rules in other species, as in the rat, mouse, and cat. In connection with Maximow's views, it is to be borne in mind that in his opinion the primitive lymphocyte is the common ancestor of all the leucocytes as well as of the defensive cells of the tissues. In a recent communication (1917) he states that lymphocytes grown *in vitro* in a plasma derived from the bone marrow develop into myelocytes.

The so-called oxidase or peroxidase methods for the demonstration of the leucocytic granules supply a new means of approach to the study of the blood cells inasmuch as they provide microchemical reactions whose results appear to depend upon fundamental biological characters of the cellular constituents acted upon by them. Whatever may be the nature of the reacting substance or substances concerned, it is commonly accepted that these methods serve to differentiate the marrow cell from the lymphocytic cell series, and they have become more and more used for this purpose. But it is believed that the differentiation can be pushed a step further so that it becomes possible to subdivide the granulocytes into two groups, the neutrophil and eosinophil showing in common a characteristic brown color reaction toward such a reagent as benzidine, while the basophil exhibits a fundamental difference from these two types through its failure to show such a color change under parallel conditions.

Upon this question of the reaction of the basophilic granule toward oxidase or peroxidase reagents, some difference of opinion is evident in the literature. As far as the tissue mast cell is concerned, Kreibich states that the granules do not react to the Winkler or benzidine solutions. Fischel, on the contrary, obtained a highly selective staining of the granules with a toluidine solution of relatively high acidity. He believed the reaction to be due to a heat-resistant or "pseudo-

peroxidase." Schultze, who first pointed out the practical usefulness of the Winkler method and popularized its use, is quoted by Loele (1912-13) and Pappenheim (1912) as having obtained a negative reaction in the tissue mast cell, but he states in one paper (1909, *a*) that in sections of the skin he obtained a strongly positive result. Unna found that the granules exerted a strong oxidative action on his reagent, the leuco base of methylene blue. He is quoted by Loele (1912-13) and Pappenheim (1912) as having obtained a positive reaction with Winkler's reagent. Loele (1910) obtained only negative reactions with alpha naphthol. He states (1911) that when amino groups are added to the benzene ring, reagents may be obtained that give a black reaction with the basophilic granule, but he considers this non-specific since it is given also by mucus. Personal observation has led to the conclusion that the basophilic granule of the fully differentiated mast cell does not react positively to alpha naphthol or to benzidine, although it is possible that in occasional cells of mast cell type there may be some evidences of a positive reaction in a few of the granules.

The present study has been concerned particularly with the basophil of the blood stream. Some of the writers reporting upon the use of various reagents fail to make specific mention of this cell, thus including it, at least by implication, in the general group of positively reacting forms. In other instances definite statement as to its reaction is made. Schultze (1909, *b*) obtained a positive reaction with the Winkler method and with his own modifications of it, and Kreibich obtained the same result both with the Winkler and with benzidine solutions. Dunn also reports a positive reaction with the Winkler method, but his statement that the reaction is weak contrasts with that of Kreibich, who concluded that the basophils in two cases of myelogenous leucemia studied by him gave the strongest reaction of any of the cell forms present. Pappenheim and Nakano obtained varying results with different reagents. In spite of some indications to the contrary in the tabulated results, they conclude that the reaction is positive and regard the negative reactions observed in some instances merely as evidence of the insensitiveness of the reagent that had been used. Nakano, in a subsequent report of a comparative study of various methods, makes no mention of the hemic basophil in any of the animals studied. He reports a negative reaction on the part of mast cells found in the spleen. It is not clear whether he is speaking here of the blood or of the tissue type, but it is probable that the latter is referred to.

In previous reports of the reactions obtained with an alpha naphthol and with a benzidine method for the demonstration of the leucocytic granules, incidental mention has been made by the author (1916, 1918-19) of the fact that the basophilic granule does not react toward either of these reagents as do the granules of the neutrophil and the eosinophil. The conclusion is supported in a recent paper by McJunkin and Charlton. These statements were made on the basis of the results obtained by the use of reagent solutions made up with 40 per cent alcohol followed by washing of the preparations in water. It has long been known that the basophilic granule is, to a varying degree, water-soluble. This

solubility is said to vary in different animal species and possibly within the same species under varying conditions of health and disease (leucemia (?)). The granule of the cell type found in the blood is more soluble than that of the tissue type. Maximow in particular emphasizes the practical importance of this water solubility as determining the precautions necessary to the successful study of the cell. In his earlier work he followed Michaelis in the avoidance of any exposure of preparations to alcoholic solutions of less than 50 per cent strength but later adopted a strength of 75 per cent alcohol as the least to which the cell could safely be exposed.

In connection with the matter of the water solubility of the gamma granule, it should be pointed out that the authors quoted above have based their conclusions as to the reaction of the cell upon the study of preparations treated with aqueous or with weak alcoholic reagents and washed with water. While such a disregard of the granular solubility would not materially affect the results obtained with the tissue basophil, it would, on the contrary, be expected to interfere more or less seriously with the certain identification of the leucocytic type. Negative findings based upon the use of such reagents must at least be accepted with caution. On the other hand, the usual experience with methods of this sort would indicate that 75 per cent alcohol would not be likely to prove a suitable solvent on account of its probable inhibiting action upon the reacting substance or substances demonstrable by these reagents. Such a solution of benzidine can be used, however, and it seems to give a definite and specific granule staining, although the reaction is somewhat retarded and is best secured through the use of solutions that have been allowed to undergo a certain amount of spontaneous oxidation. Such a solution makes it possible to study the benzidine reactivity of the basophilic granule under conditions calculated to avoid destruction of the granular substance by the staining or washing fluids. Thionin counterstaining gives a striking differentiation of the non-reacting gamma granules as well as distinct nuclear, cytoplasmic, and platelet staining.

Fresh films from a considerable series of cases of myelogenous leucemia were studied.¹ The technique of the benzidine method used

¹ The material necessary for carrying on a study of the hemic basophil as stained by a method founded upon the above considerations was supplied largely by Miss A. L. Gibson, Superintendent of the Huntington Memorial Hospital of Harvard Medical School.

in the staining of these films has been slightly altered from one previously reported, the principal point of difference lying, as indicated, in the avoidance of exposure of the preparations to water or to alcohol of less than 75 per cent strength. 40 per cent alcohol is not entirely destructive to the granules, while 50 per cent alcohol preserves most of them, but their complete preservation can only be assured through the use of the higher concentration.

Method.

The fresh film is fixed in a freshly prepared mixture of one part of neutralized formaldehyde (40 per cent of the gas) with nine parts of 95 per cent alcohol. This fixation is not absolutely necessary, but sharper pictures are obtained with it. The preparation is washed in 75 per cent alcohol and covered in a shallow slide dish with a solution of benzidine in 75 per cent alcohol. The benzidine is made up in 0.5 per cent strength and the solution receives 0.2 per cent of hydrogen peroxide, U. S. P., of tested activity. It is best to allow the benzidine solution to stand at least 24 hours before use in order that a certain amount of spontaneous oxidation may take place, this apparently rendering it more susceptible to further change by the granular substance. Spontaneous oxidation begins in a few hours and is evidenced by a slight brownish coloration of the originally colorless fluid. The solution keeps for weeks. The granular reaction takes place somewhat more slowly than it does when 40 per cent alcohol is used as a solvent and the granules are not so heavily stained, although, as already stated, there does not appear to be any failure in the specific nature of the reaction nor does it appear that the solution fails to stain any granules that would be stained by the 40 per cent reagent. A good granular staining may be evident after 5 minutes, but it has been customary to allow a reaction time of 10 minutes. After being well washed in 75 per cent alcohol the film is stained with thionin. This is made up, as recommended by Maximow (1913), by adding two drops of a 2 per cent aqueous solution of sodium carbonate to 10 cc. of a stock saturated solution of thionin in 75 per cent alcohol. The solution should be allowed to stand 24 hours before being used. It keeps for 2 or 3

weeks. There is no danger of overstaining. 5 minutes has usually been allowed. The film is washed thoroughly in 75 or in 95 per cent alcohol and dried. Balsam neutralized with sodium carbonate has been used for mounting cover-slip preparations, but neutralization is not necessary. The method is applicable to frozen sections.

Results with the Benzidine-Thionin Method.

The method gives a distinct and strongly differential staining of the three granule types of human blood (Fig. 1). Neutrophilic and eosinophilic granules are brown, while, in strong contrast, the basophilic form shows a color varying from a deep opaque purple-red to a more transparent violet-red. This well known metachromatism of the gamma granule is brought out much more sharply by thionin than by the eosinate stains in common use. The color range of the thionin-stained granules is greater in some cases of myelogenous leucemia than in others. Thus in the films from one patient the granules may be almost entirely of the darker staining type, the metachromatism manifesting itself only to a minor degree, while in preparations from another case they may show marked color differences. Here some of the basophils carry granules of one color type, others show only those of the contrasting tint, while again minor variations in the color reaction appear in the individual granules of a single cell. The darker staining granule is apt to be rather smaller than the lighter staining one. It does not usually exceed the alpha granule in size and may be much smaller. The paler type shows a tendency toward occurrence as large round, oval, or irregular, often angulated bodies as large or even much larger than the alpha granule. They may show a central relatively unstained area and in general appear to be swollen and poor in tingible substance. The deeper stained granule appears usually in cells of the smaller diameters (Fig. 2). The deeply stained nucleus of such a cell often suggests that of a lymphocyte, or it may be made out dimly as a lobulated mass simulating, though never reproducing closely, the polymorphous nucleus of a neutrophilic leucocyte. It exhibits in general a tendency toward the formation of the bizarre forms illustrated by Weidenreich. The larger granule, on the other hand, is found in

cells of larger diameter with more faintly stained nucleus, which may be multilobulated but is again different, particularly in its lesser chromatin content, from the type usually connoted by the adjective polymorphous (Fig. 3). The granules are apt to be less numerous and more widely spaced in the cytoplasmic body than they are with the smaller type.

Possible Causes for the Variations in Staining of the Basophilic Granules.

The idea occurs at once that these variations might simply be the result of a greater or less flattening out of the cell by the varying pressure to which it may have been subjected as the film was made. It is unquestionable that a certain amount of variation can be produced in this way, as can be shown in any blood film by whatever method it is made or stained, but it is not believed that the varying appearances described can be due to artifact to more than a minor degree. Basophilic cells of sharply contrasting appearance may be found side by side in thin fields with widely spaced cells, while on the other hand thick fields of closely crowded cells still allow a differentiation of the two general types.

Another question suggesting itself is whether the thionin coloration of the granules may not merely be superimposed upon the brown of a primary though possibly weakened benzidine reaction. With this possibility in mind, films were studied before and after thionin counterstaining and the findings in individual cells at the two stages compared. Cover-slip preparations were divided into sets of small squares by lines drawn upon the reverse side with marking ink, the films were treated with the benzidine solution, mounted in 75 per cent alcohol, and the various fields plotted. The preparations were then counterstained and the cells as finally stained compared with the provisional classification of the first charting. In the preparations thus studied a sufficient number of basophils was encountered to establish the conclusion that the basophilic granular material showed no evidence of a brown coloration after benzidine. In the later preparations the basophils could be picked out with some degree of confidence in the first plotting as colorless bodies with indistinct nuclear outlines and scattered vacuole-like markings

slightly if at all more refractive than the cytoplasmic substance in general. Indeed, some of the granules are evidently overlooked under these conditions, since cells in which, during primary plotting, only a few scattered granules could be made out might present in the final picture a cytoplasmic body crowded with the characteristic basic stained bodies. A parallel plan was followed for the tissue basophil and here again no benzidine coloration could be made out in the fully differentiated cells.

Variation of Granule Types in the Individual Cell.

It is well known that the myelocytes of normal bone marrow as well as those found in the circulating blood in myelogenous leucemia may show a mixed granulation; that is, the presence within a single cell of granules exhibiting contrasting staining affinities. In man, the associated forms are almost always eosinophilic and basophilic, although the coexistence of neutrophilic and basophilic types has been recorded (Bloch and Hirschfeld, Engel). In the guinea pig and rabbit basophilic granules may also occur in association with the pseudo-eosinophilic or beta type. This heterochromatic granulation, as it was termed by Levaditi (1902), has been the subject of considerable discussion, the interest centering in the question as to whether the basophilic bodies are to be considered as actual gamma granules or as neutrophilic, eosinophilic, or pseudo-eosinophilic forms with a peculiar variation in their staining affinity dependent upon incomplete differentiation or upon degenerative changes in the proper granules of the cell. The early discussion was complicated by a confusion of terms, and it was not always clear whether a writer used the word basophilia in its general sense or in its restricted meaning as applied to the peculiar metachromatic basophilia of the true gamma granule (Arnold).

Myelocytes containing eosinophilic and metachromatic basophilic granules are not uncommon in some cases of myelogenous leucemia. They seem to occur particularly in cases showing the wider variations in the appearance of the true basophils. The two granular types occur in varying proportions in different cells and the containing cells vary considerably in size. The larger examples usually contain a pre-

dominant number of eosinophilic granules (Fig. 4), and one gains the impression that as the relative number of gamma granules increases the cell becomes smaller. Thus the smallest cells encountered contain numerous gamma granules with only an occasional alpha form embedded in the compressed field of closely crowded basophilic masses (Fig. 5). The nucleus is most often of myelocytic type, definitely so in the larger forms, but as the cell becomes smaller the nucleus likewise diminishes in size and takes on a heavily stained, shrunken appearance with outlines often masked by the apposed or overlying granules. Where most clearly made out it often shows an irregular outline with shallow indentations or small bud-like protrusions of its substance. Occasionally it may suggest the polymorphous configuration, but the internal nuclear structure is not that of the neutrophilic cell. The appearance of neutrophilic and basophilic granules in the same cell is certainly very rare, but at least one cell has been observed that seems unquestionably to be a polymorphonuclear neutrophil containing a few typically metachromatic basic staining granule masses. Its proper neutrophilic granules have partially disappeared (Fig. 6). In the eosinophilic-basophilic cells the individual granules are usually of pure type, that is, they are definitely brown-stained or thionin-stained, but occasionally cells are encountered in which the individual granules may show a mixed staining reaction. In one cell, as illustrated in Fig. 7, rupture of the cell membrane has allowed a wide dispersal of the granules so that each may be closely examined. It will be seen that granules of three types appear. There are granules of unquestioned alpha type though of variable size and variable depth of color; others that again show variations in size and staining reaction are of undoubted gamma type; while in a third type an underlying brown color appears to be more or less replaced by a metachromatic thionin staining. Here the basic staining substance appears as a scarcely perceptible peripheral darkening of the body in a part or the whole of its circumference or as a definitely reacting area or sector showing variable metachromatic tints and involving little or much of the granular mass. It looks as though the basic staining substance makes its appearance at some focus in the granule and extends thence to involve the whole substance. The purely basic stained granules vary

from minute fragments to large irregular bodies and show a variable staining reaction. The same changes are present in the granules of the cell represented in Fig. 8, but they are not so plainly made out and the cell appears not to be in so good a state of preservation.

Distinguishing Characteristics of the Basophilic Granule.

In correspondence with its failure to react toward benzidine, the basophilic granule differs in other respects from the neutrophilic and eosinophilic types. Its characteristic water solubility has already been mentioned. This may be only an exaggeration of a property present to some degree in the other two forms, but it is still sufficiently peculiar to constitute a distinct type characteristic. It is also resistant to drying. The benzidine-active granules begin to show a gradual loss of their property of benzidine coloration within a comparatively short period after the film is made. The neutrophilic granule shows this change first and most strikingly, but the alpha form also loses its reactivity after a longer period. In films of normal blood 6 months old the neutrophils appear entirely devoid of granules under the benzidine-thionin stain, while the eosinophilic granules are colorless or at the most show but doubtful traces of a brownish tone. In the occasional basophils found in such a preparation, however, the gamma granules show a staining reaction slightly if at all altered from that characteristic of the fresh film. Like results are seen in heated preparations. When fresh films are subjected to dry heat of a temperature averaging 150°C. the benzidine reaction of the neutrophilic granule is quickly destroyed. After 10 minutes no trace of the neutrophilic granules can be seen in benzidine preparations, while the eosinophilic granules react rather feebly. After 20 minutes the eosinophilic granules also appear inert. Meanwhile the basophilic granules have shown no change beyond a possible slight lowering of their color tone, and even after 30 minutes at the given temperature these cells have a natural appearance, although the granules show a lowered color tone and an added emphasis of the central comparatively unstained portion often seen in fresh preparations.

Resistance is shown also to treatment with acids and alkalies. Applied to an unfixed or even to a fixed blood film, a 1 per cent solution of potassium hydroxide in 75 per cent alcohol destroys the neutrophilic granules practically at once so that no trace of them can be found upon subsequent benzidine staining. The benzidine reactivity of the eosinophilic granule is also destroyed, although the granule mass itself can still be made out as a colorless body exhibiting the characteristic refractivity. The gamma granule resists much longer exposure. The same resistance is evidenced toward acid solutions. Short exposure to a 1 per cent solution of acetic or lactic acid in 75 per cent alcohol changes or destroys the reactivity of the neutrophilic and eosinophilic granules without changing the appearance of the basophilic type. Acid and alkaline solutions show the same action when applied to frozen sections of formaldehyde-fixed tissue containing numerous basophils of the tissue type in association with polymorphonuclear neutrophils.

It is well known that variations in the hydrogen ion concentration of the media may affect the granular staining brought about by the so called peroxidase reagents. These variations may alter the color of the stained granules or they may result in total failure of the reaction. The findings in blood films exposed for varying intervals to weak acid solutions suggest that the result is due to a progressive change in the granular substance, and there is a certain amount of evidence that at some stage in the course of its disintegration the granule may become basophilic and inactive toward benzidine. When fresh films are exposed to the acid solution for 30 seconds, washed in 75 per cent alcohol, and stained with benzidine and the stock non-alkalinized thionin, the neutrophilic granules show a variable color reaction. In fresh films the neutrophils are usually filled with a closely crowded mass of opaque greenish blue granules. In preparations 2 or 3 days old the changes are generally more extensive. Many of the granules fail to stain, while the forms that are still demonstrable show wide variation in staining reaction. A few may still show a slight brownish coloration, many are of a dull greenish blue, while scattered through the cytoplasm there appear occasional bodies of about the size or of slightly larger size than the neutrophilic granule which exhibit a metachro-

matic thionin coloration not to be distinguished from that of the granules in a typical basophil (Fig. 9). From their arrangement and general appearance it seems clear that these represent chemically altered neutrophilic granules. The appearance of these cells recalls that of the neutrophil (Fig. 6) found in the blood from a patient suffering with leucemia. Experimental work founded upon this observation is in progress.

DISCUSSION.

The basophilic granule exhibits certain physical and chemical reactions that appear to be different from those characterizing the neutrophilic and eosinophilic types. As compared with them it seems chemically more stable while biologically inert. Study of the cell forms found in the leucemic blood of man suggests strongly that the metachromatic basic staining granular substance arises in some way as the result of changes taking place in the granules of what may be termed the essential or true leucocytes, or myelocytes. The progress of the changes leading to its appearance may be followed in the cells showing the heterochromatic granulation above described. In such cells as the ruptured eosinophilic myelocyte illustrated, the benzidine-active substance appears to undergo a gradual change into a substance no longer endowed with this property of reacting in characteristic manner to a benzidine solution. This change is taken to imply a fundamental alteration in the nature of the granular material. The concomitant acquisition of a basic staining property of peculiar type serves as further evidence of the chemical alteration in progress. In the cells as a whole there occur all stages of the conversion of an eosinophilic cell filled with active granules into cells whose granules appear entirely inert toward benzidine. These inactive granules are water-soluble but resistant to physical and chemical agents destructive to the benzidine-active granular substance. Accompanying the granular changes there appears to be a progressive degenerative change in the nucleus. It shrinks, becomes more compact and more heavily stained, and takes on a crumpled or shrivelled appearance. The end-product becomes a small cell with a gnarled nuclear mass and a thin cytoplasmic envelope crowded

with the basic staining remains of its original granules. In other instances it may be that the degenerative change goes forward less rapidly, or, in other words, the cell is able to maintain a metabolism more nearly equal to the normal so that despite the progress of the granular changes the cell as a whole secures a better approximation to the normal evolutionary course. Under these conditions the nuclear appearance may simulate more or less closely that of the true leucocyte, while there may remain a considerable amount of cytoplasmic substance whose granules tend to be relatively fewer than in the first case and more lightly stained. This is the type usually found in normal blood, while the smaller type is more typical of leucemic blood.

Whether or not the basophil is a true granulocyte has been questioned repeatedly. Harris while studying the tissue mast cell was impressed by the fact that its granules react toward dyes just as mucus does, and after trial of various stains, including the special methods of Mayer, he concluded that the granular material actually consists of mucin, whence he called the cell a "mucinoblast." He describes cells with similar granules which he had observed in the lymphatics of inflamed tissues and in the blood and believed that these forms arise from the "hyalin" cells. A few years later Pappenheim expressed his belief in the mucinous nature of the basophilic granule and the view has been urged repeatedly by him and his followers. Like Weidenreich, he concluded that the mast cell may represent a degenerated lymphocyte, but while the latter derived the basophilic granules from particles of nuclear material, Pappenheim regarded them as the product of a mucoid degeneration of the spongioplasm, and from a further study of their staining reactions arrived at the conclusion that they are in fact made up of an alkaline mucolipoid (Pappenheim, 1906, 1912). Since the early work of Ehrlich it has been generally accepted that the myelocytic granules show in the first stages of their development a basophilic staining reaction. It is usually accepted that this basophilia is not of the metachromatic type characteristic of the true gamma granule. Pappenheim, however, regards the basophilic granules of normal blood and bone marrow as immature examples of the alpha or of the special or beta granules. He considers that the degenerated lymphocytic type of the cell occurs in the blood of man only in leucemia. For Pappenheim and his followers, therefore, three possibilities are given as to the significance of the basophilic granules as they occur in the cells of the blood and bone marrow of man and other vertebrates: they may arise through degenerative changes in a non-granular lymphocyte (such a cell does not, it must be remembered, represent, in the so called monophyletic view of this school, a series distinct from the granule-bearing cells); they may represent an early developmental stage of the alpha or beta granules, or "an aplasia, abortive formation, or degeneration of the

'ripe' granules;" and, lastly, "there may be a combination of both the above possibilities in that the granules appear in such lymphoid cells as were originally destined to the formation of oxyphilic granule cells" (Pappenheim and Szécsi).

The contention that the basic staining granules occurring in the cells of mixed granule type are early undifferentiated forms of one or another granular variety would not seem to apply to the preparations described in the present paper, for it is well known that the various peroxidase methods are capable of demonstrating a positive granular reaction in cells of the myeloblast stage, where the usual stains fail to show any evidence of granule formation. All the evidence at hand indicates that benzidine is fully sensitive enough to accomplish this result. It is, therefore, hardly possible that definitely formed granules of essential type could fail to give a positive benzidine reaction.

Weidenreich attacked the whole plan of leucocytic classification based upon staining reactions instead of upon morphological considerations and objected in particular to the prevailing conception of the hemic basophil as a normal leucocyte. He considered this cell as different chemically and physiologically from the other leucocytes in all animals except the guinea pig. In the latter he recognized a true mast leucocyte with specific granules and a regular course of development from a mast myelocyte of the bone marrow. In all animals other than the guinea pig he regarded the basophil as a degenerated lymphocyte whose granules were derived, directly or indirectly, from particles of nuclear material that had become broken off and extruded into the cytoplasm. In support of this view he urged his failure to find a centrosome in any basophils except those of the guinea pig, and the further argument that the morphological peculiarities of the nucleus are not those of the true leucocyte but rather those of a degenerating nucleus of lymphocytic type. Downey (1912) believes that the tissue mast cell in the cat is developed from the lymphocyte and that its granules are derived from particles of nuclear material. Blumenthal concluded that the basophilic leucocyte is derived from the same finely granular basophilic myelocyte that gives rise to the neutrophilic and eosinophilic series, the last two representing the normal lines of development, while the basophil is a pathological form appearing in numbers only under conditions of disease. Gulland suggested that the tissue mast cells are leucocytes specially differentiated from long residence in the connective tissues and that the basophils of the blood and bone marrow may be examples of such connective tissue cells which have escaped into the blood stream.

Functionally, the basophil seems to be inactive. It is encountered only infrequently in normal blood, and the laws governing its increase in the blood or tissues

under pathological conditions do not appear to be those obeyed by the other blood cells. Fahr concluded that it is not phagocytic and that it appears to take no part in the ordinary phenomena of leucocytosis, although he concluded, on the basis of its disappearance from the peritoneal cavity after the injection of virulent organisms, that it may exhibit a negative chemotaxis. He showed that a negative chemotaxis common to all the leucocytic forms may become positive after immunization in all types except the basophilic. Routine clinical and laboratory experience would seem to offer little in favor of the investment of the cell with properties of phagocytosis or other defensive activity.

In studying septic and aseptic inflammations in animal tissues Maximow (1904, 1905) failed to find any evidence of phagocytic activity on the part of the basophilic leucocyte, nor was there any clear indication of a direct response on its part to bacterial invasion, although the cells are present in numbers in the marginal tissues of a purulent focus. He believed that both basophilic forms give off their granular material into the surrounding tissues either by simple extrusion of the granules or as the result of a complete disintegration of the cell. This disintegration of the mast cell in an inflammatory area may be extensive, and the granular material may be taken up by the phagocytic leucocytes or by polyblasts which attack the degenerating cells, or it may be deposited, directly or indirectly, in fixed connective tissue cells. He describes polyblasts showing a generalized metachromatic staining of cytoplasm and nucleus, this appearance being accompanied by obvious degenerative changes in the cells. In the scar tissue resulting from an inflammatory process the mast cells still appear, sometimes in considerable numbers. He is inclined to regard the granular material as a nutritive substance that may be concerned in some way with tissue metabolism or with defense processes. Greggio likewise believes that the tissue mast cell may be a sort of unicellular gland capable of existing in the tissues in an undifferentiated resting stage until aroused by local nutritional disturbances such as occur, for example, in inflammation, when it may become active and take up its specific function of elaborating granular material and discharging it into the surrounding tissues. Harris and others have considered it possible that the granular material thus discharged may be bactericidal, but there is no evidence to support this view.

The conception of the gamma granule as the product of degenerative changes is in harmony with observations recorded in the literature as well as with certain facts of common experience. Maximow (1905) describes the appearance in old pus collections of a granular material having the tinctorial characters of the metachromatic granules, and, more significantly, notes the presence of metachromatic granules in the cytoplasm of large actively phagocytic cells scattered about an abscess cavity in rabbit tissues. He is unable to explain their appearance here but considers it possible that they may be a synthetic cellular product derived primarily from some constituent of pus. It would be, perhaps, no less reasonable to regard them as the product of the disintegration of cellular material that is undergoing digestion within the phagocytes. The disintegrating

mast cells described as occurring in the margins of an abscess and the metachromatic change accompanying the obvious breaking up of the polyblasts may likewise be interpreted as evidences of the degenerative changes that are taking place in and about such areas. In this connection it is of interest that Harris describes a "smooth muscle mast cell" as occurring in the muscular wall of a tubercular intestine and in the vascular media in tumors. Here obvious degenerative changes in smooth muscle cells were accompanied by the appearance in the cell cytoplasm of numerous small metachromatic basic stained granules. Wolff found that after the injection of spermatozoa into the peritoneal cavity of the guinea pig the phagocytic mononuclear cells closely resembled mast cells, their cytoplasm being filled with granules regular in size and arrangement and exhibiting a definite metachromatic staining reaction to methylene blue although not to thionin or to cresyl violet. He showed that the granules represented bits of the disintegrated spermatozoa. These findings suggest that the property of reacting metachromatically toward certain basic dyes may be a general property of disintegrating cellular material. Incidentally, they emphasize also the weakness inherent in any method of granular classification founded merely upon staining reactions. The objection may apply to the concept set forth in this paper, but despite our ignorance as to the final significance of the granular reaction provoked by the so called peroxidase reagents, it is believed that the conclusions drawn have in their favor demonstrable granular differences that are dependent upon variations in the granular substance more fundamental in their nature than any that can be made evident through the single factor of a varying reaction towards the aniline dyes.

Occasional attempts have been made to produce experimental basophilia or basophilic leucocytosis. Levaditi (1902) reports basophil counts of 10 to 13 per cent in rabbits and guinea pigs following the subcutaneous injection of bacterial toxins and the intraperitoneal injection of a specific hemolysin. It is noteworthy that the basophil increase shown in his charts occurs comparatively late, and the question is suggested whether this increase may not constitute a function of degenerative rather than of regenerative or proliferative changes in the blood cells. Such an explanation may apply also to the few other instances of experimental basophilic leucocytosis that have been reported. Thus, Pröschner (1904), in experiments carried out with Pappenheim, claims a basophilic leucocytosis in the rabbit as the result of the injection of a hemolytic poison, "phrynolysin," and several years later (1909) he again observed it in the rabbit following the injection of supposed cultures of an ultramicroscopic smallpox virus. Pappenheim and Szécsi obtained a basophilic leucocytosis in the rabbit with saponin or saponin combinations, and they quote Kasarinoff as having found it in the bird after saponin injections. Two facts are of interest in these reports. First, the agents found capable of producing an experimental increase of the basophils are usually substances having a direct destructive action upon the blood and blood-forming organs, and, second, it is noticeable that with the increase in the basophils there occurs a rise in the numbers of the eosinophils or pseudo-eosino-

phils. Thus, in two rabbits that had received the smallpox virus the basophils increased to counts of 18 and 16 per cent (the stated normal is 4 to 8 per cent), while the polymorphonuclear eosinophils rose to 4 and 3½ per cent respectively (the normal is given as 0 to 0.57 per cent), and pseudo-eosinophilic myelocytes made their appearance in the circulating blood, rising to levels of 7 and slightly over 7 per cent. In a recent report dealing with experimental benzol poisoning in rabbits, Weiskotten and Steensland record the occurrence in animals of a definite basophil increase. This appears comparatively late and may conceivably be the expression of a degenerative process set up in the blood-forming tissues.

The most striking instances of experimental basophilic leucocytosis in the literature were obtained by Schlecht in guinea pigs inoculated intraperitoneally with foreign sera. In animals dying after repeated intraperitoneal injections of horse or sheep serum he claims to have observed marked increase in the basophils, these cells reaching counts of even 25 to 28 per cent of the total leucocytes. He was interested primarily in the study of eosinophil variations and believed that he could find some evidence of a reciprocal variation in the numbers of eosinophilic and basophilic cells, the two types varying in inverse ratio. His animals showed at first a decrease of the eosinophils and basophils; this was followed by a fluctuating rise in the eosinophils with a variable but distinct basophilic increase. During the last 3 or 4 days before death occurred the eosinophils showed a marked drop in their numbers and, in one animal, complete disappearance, while the basophils rose more or less abruptly to the unusual levels quoted. The cause of death in these animals is not made clear.

There is on record, then, a certain amount of experimental evidence that an increase in the eosinophils or pseudo-eosinophils of animals may be accompanied by fluctuations in the number of basophils. If the suggested relation between the cell types holds it must, however, depend upon some factor not always operative in conditions capable of exciting an increase in the eosinophils of the circulating blood, since there is but little evidence in its favor in the eosinophilias commonly encountered in man aside from the eosinophilia occurring in leukemia. In three cases of trichinosis, of which two had eosinophil counts of 23 and 26 per cent, while the third, a moribund patient, presented a terminal drop in the count to 1 per cent, no increase in the basophils was found. No increase was seen in two cases of bronchial asthma recently under observation, both of which presented slight eosinophilia. Slightly more suggestive are the findings in a rabbit suffering from a well developed case of aural mange (*Demodectes cuniculi*). Here a count of 1,000 cells showed 4.6 per cent

eosinophils and 6.6 per cent basophils. The latter figure can hardly be considered as constituting a distinct basophilia, since the normal range of the cell in this animal is usually accepted as from 2 to 5 per cent, but at least it is a high normal. One case of parasitism in man has been seen in which there appears to have been a coincident rise in eosinophils and basophils. The case was one of hookworm infection and the differential count (500 cells) showed 13.8 per cent eosinophils and 4.6 per cent basophils. The eosinophilia obtained by Herrick in guinea pigs treated with *Ascaris* extracts was accompanied by fluctuations in the basophil count. The highest level recorded is 7.5 per cent.

The question as to the nature of the hemic basophil is, of course, bound up intimately with that as to the existence in the bone marrow of the ancestral forms of the cell. Consistently with their denial of the existence of a true basophilic leucocyte, the followers of Pappenheim fail to find a true basophilic myelocyte in the bone marrow (Benacchio, Kardos, Pappenheim (1912)). Weidenreich likewise denies its existence except in the guinea pig. Maximow (1913), on the contrary, finds both forms in various animal species. Both are, however, more infrequent than cells with eosinophilic or neutrophilic granules and in the bone marrow the leucocytic phase predominates numerically over the myelocytic. Maximow's findings are supported by Downey (1914) and by Ringoen for the rabbit and guinea pig. It is to be noted, however, that the basophil is stated to exhibit a heteroplasmic type of development as contrasted with the homoplasmic type characteristic of neutrophilic and eosinophilic cells. While the last two multiply almost always through the mitotic division of cells already possessing granules, the basophilic leucocyte is developed characteristically without mitosis from a non-granular cell through the gradual appearance of the basophilic granules in its cytoplasm and subsequent nuclear changes toward the usual leucocytic type. These changes are not considered as degenerative. Downey describes them as occurring in the eosinophil series also.

Realizing the difficulties imposed in the way of a full acceptance of his views by the doctrine of a purely heteroplasmic genesis of the basophilic leucocyte, Maximow made the point the subject of study in a number of animal species. He finds (1913) dividing mast myelocytes in the guinea pig, rabbit, rat, and rarely in man. He admits that it requires long search to demonstrate an example in human bone marrow, and it may perhaps be said that the figure reproduced as an example of a dividing cell is not particularly convincing. On the other hand, they are said to be frequent in the guinea pig. It will be recalled that Weidenreich came to the conclusion that this is the only animal species of several examined in which the existence of a true basophilic myelocyte with derivative leucocytes could be proved. In this connection it is further of interest that

Levaditi (1902) described the "ovoid granule" of the guinea pig as a distinct type that is peculiar to this animal and found in no other. He finds evidence for this view in its greater resistance to solvents (Maximow also considers it relatively insoluble in water) and in the fact that it is stained by the tri-acid mixture, which fails to stain the basophilic granule encountered in other species. Under benzidine-thionin the basophilic cell of guinea pig blood sometimes suggests the cell of mixed granule type found in the leucemic blood of man rather than the true basophil of human blood. A few of the granules may take a dull brown color and the thionin tints of at least a portion of the others may seem to be combined more or less obviously with a brown coloration as though superimposed upon it. It is possible that the basophil of the guinea pig possesses granules that are relatively better preserved than those of other species, and in this fact may lie an explanation for its having been singled out by different writers as peculiar in one or another respect. When thus distinguished, qualities have been attributed to it more like those of the active leucocytes than those of the basophils of other species.

It has not been possible thus far to make any satisfactory study of the bone marrow under the benzidine-thionin stain, and the discussion of this vital phase of the question must be deferred until there may be found some method applicable to paraffin or other thin sections that will be more satisfactory than any available at present. Films of the fresh bone marrow are unsatisfactory for finer cytological study, since it has been impossible in such preparations to avoid a general sprinkling of the slide with free granules, and these adventitious bodies make a study of individual cells very difficult.

Another line of inquiry into which it has not been possible to go is that of comparative hematology. The field is large and considerable confusion appears to prevail in it, but it is possible that a study of the basophilic forms encountered at different levels of the evolutionary series might throw some light upon the present problem.

It is stated by Werzberg that the basophil does not appear in the teleost fishes except in the form (*Carassius auratus*) possessing the most highly developed blood picture. The eosinophil appears much lower in the scale. Once developed, however, the basophil appears to become relatively much more frequent than it is in the higher forms. Thus, Klieneberger and Carl find in the frog (*Rana esculenta*) 23 per cent of basophilic forms, 6 per cent of eosinophilic, and 26½ per cent of non-granular polymorphonuclear cells. Goodall found the basophils present "in considerable numbers." The occurrence of a high basophilic ratio is supported by the findings in a few preparations from material that has come to

hand incidentally during the course of the present study. The basophilic percentage in a tortoise (*Emys lularia*) is quoted by Werzberg as from 6 to 8 per cent. The benzidine stain has shown a striking predominance of the basophilic forms in this animal, comparatively few of the granulocytes showing the brown benzidine reaction. The basophilic percentage obtained for the common fowl by various workers whose results are tabulated by Burnett varies from 1 to 4.3 per cent, but a much higher figure is indicated in films stained by the present method.

The apparent discrepancies in the various findings for the blood of the lower forms probably depend upon the marked water solubility of the basophilic granular substance in the blood concerned. There is a striking contrast in the appearance of films of chicken blood stained by the benzidine-thionin and by the usual eosinate methods. There is some indication that occasional cells in the blood of these lower forms may show a mixed granular reaction.

These fragmentary observations upon the blood of the lower animals serve to warn against too dogmatic a conclusion as to the interpretation to be placed upon the basophil. It is not to be questioned that a cell type present in any species in such predominating numbers as those quoted above may well be suspected of having a real functional significance, at least for the species concerned. Another consideration based upon the facts of comparative hematology is not to be lost sight of. While the number of basophils relative to the other white cells of the blood is uniformly small in mammals, it is fairly constant within each animal species. This may mean only that the leucocytes or marrow cells within the given species are subject to a fairly constant set of conditions leading to the appearance in them of a peculiar degenerative change, but, on the other hand, it may indicate that the basophil is not a dead or dying cell but a functioning blood unit possessing some obscure rôle in the general body economy. But while such a possibility is not to be disregarded, it is still believed that the evidence thus far at hand points in the main toward an interpretation of the cell as a pseudoleucocyte, and it is felt that the basophilic granule as found in mammals and particularly in man is not homologous with the neutrophilic and eosinophilic types and that the human basophil is not to be considered as a true leucocyte in the usual meaning of the term.

CONCLUSIONS.

1. The basophilic granule of blood and marrow cells does not show the brown color reaction with benzidine solutions that is characteristic of the neutrophilic and eosinophilic granules. It differs from these types also in other important microchemical and physical particulars.

2. The hemic basophil which bears these granules is peculiar from a purely cytological standpoint, while physiologically it appears to be devoid of any functional activity comparable with that of the other granulocytes.

3. The peculiarities exhibited seem in every instance, at least for mammalian blood, best explained as evidence that the basophil is a degenerated or degenerating cell. It is probably derived from the eosinophilic cells and perhaps in rare cases from those of neutrophilic type.

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EXPLANATION OF PLATE 15.

FIG. 1. Myelogenous leucemia. A field from a blood film stained with benzidine-thionin. *a*, eosinophilic myelocyte; *b*, neutrophilic myelocyte; *c*, polymorphonuclear neutrophil; *d*, basophil; *e*, early myelocyte; *f*, myelocyte with granules of mixed type.

FIGS. 2 to 9. Isolated cells from the blood of myelogenous leucemia and from a film of normal blood treated with an acid solution before staining. Benzidine-thionin method.

FIG. 2. Basophil of smaller type with heavily stained granules.

FIG. 3. Basophil of larger type with lobulation of the lightly stained nucleus and granules of the paler type. The granules are variable in size and shape.

FIG. 4. Eosinophilic myelocyte with a few gamma granules. Early nuclear changes.

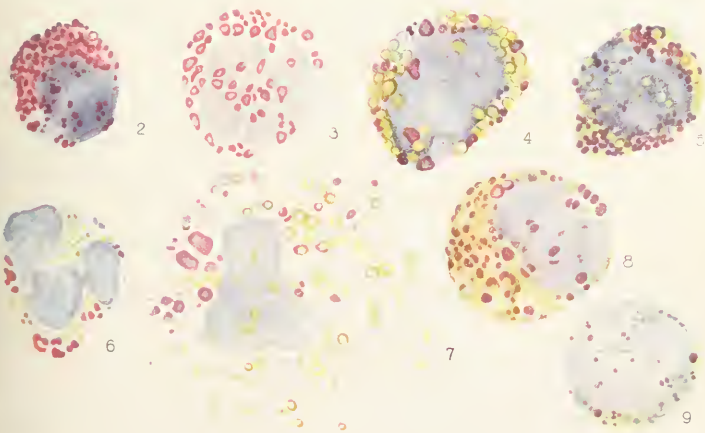
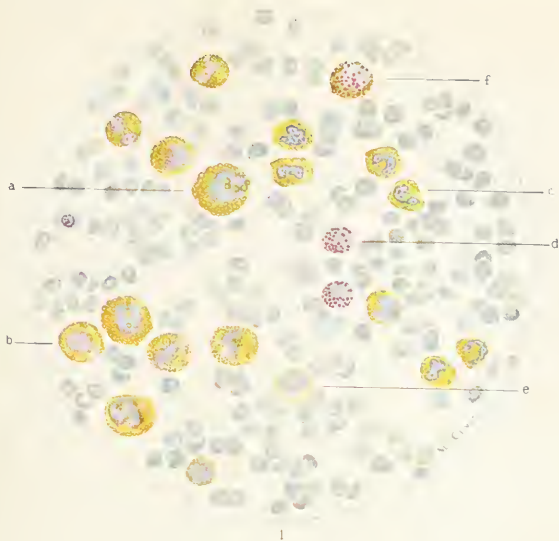
FIG. 5. Smaller cell with shrunken, more deeply stained nucleus. The gamma granules outnumber the eosinophilic.

FIG. 6. Polymorphonuclear neutrophil with basophilic granular changes.

FIG. 7. Ruptured eosinophilic myelocyte. Basic staining material making its appearance in individual granules.

FIG. 8. Myelocyte with constricted nucleus and progressing granular changes.

FIG. 9. Polymorphonuclear neutrophil of normal blood with granular reaction altered by treatment with an acid solution before staining. Scattered granules exhibit a metachromatic thionin reaction.



(Graham: Hemic basophil.)

PNEUMOCOCCUS CULTURES IN WHOLE FRESH BLOOD.

I. THE RETARDATIVE EFFECT OF THE BLOOD OF IMMUNE ANIMALS AND THE MECHANISM OF THE PHENOMENON.

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The failure to realize more fully in the field of specific immunotherapy the expectations that were engendered by the discovery of specific antitoxins for diphtheria and tetanus is due, no doubt, in large measure to the lack of definite and complete knowledge of the mechanisms of immunity reactions.

It is noteworthy that, with few exceptions, a multiplicity of theories has been evolved with respect to the identity and nature of the factors and processes operative in natural recovery from each infectious disease to which man is subject. This is true of infections by the pneumococcus, notwithstanding the fact that these infections lend themselves, to an exceptional degree, to experimental study. It would be difficult to mention a more striking example of antibacterial immunity than that conferred upon a susceptible animal by the administration of antipneumococcus serum. Moreover, some of the species of laboratory animals are highly susceptible to pneumococcus infections, while others are refractory to a corresponding degree, thus making it possible to study any type of immunity—natural immunity or active or passive acquired immunity.

The protective power of antipneumococcus serum has been ascribed to different factors by various investigators. Thus Mennes,¹ Boehncke and Mouriz-Riesgo,² and others advanced the theory that antipneumococcus serum possesses marked

¹ Mennes, F., *Z. Hyg. u. Infektionskrankh.*, 1897, xxv, 413.

² Boehncke, K. E., and Mouriz-Riesgo, J., *Z. Hyg. u. Infektionskrankh.*, 1915, lxxix, 355.

antitoxic properties, while Neufeld and Rimpau^{3,4} claim that pneumococcus immunity depends entirely upon the bacteriotropic substances of the immune serum and the phagocytic cells of the host. More recently it has been pointed out by Wright⁵ that the whole uncoagulated blood of man is, in certain instances, highly pneumococidal, and a case is cited in which 1 cc. of blood killed from 600,000 to 1,000,000 organisms. Heist and his collaborators^{6,7} have extended Wright's observations, including experimental animals. It is claimed by them that the pneumococidal capacity of the whole uncoagulated blood is a direct index of resistance to pneumococcus infection and that the virulence of the organisms is directly proportional to their insusceptibility to this killing property of the blood. Cole and his coworkers⁸ and Winternitz and Kline⁹ believe that any or all of the factors mentioned above cannot be held entirely responsible for immunity to pneumococcus infection and that an unidentified factor is essential.

The work reported in the present paper concerns the alleged pneumococidal property of the whole blood of immune animals and it is therefore desirable to state briefly a few points concerning antipneumococcus serum which are of interest in this connection. Pneumococci can be grown for an unlimited number of generations in the most potent antipneumococcus serum. This treatment profoundly affects the organisms, as shown by Stryker,¹⁰ yet there is no evidence that any of the organisms are killed by the serum. The same is true of the blood serum of naturally immune animals. Pigeons, for example, possess practically an absolute immunity to pneumococcus infections, while the fresh serum of pigeons is a good culture medium for pneumococci. The defibrinated blood of immune animals is only a slightly less favorable medium than the serum. On the other hand, pneumococci do not multiply in the blood stream of refractory animals and the intravenous administration of antipneumococcus serum sterilizes,

³ Neufeld, F., and Rimpau, W., *Deutsch. med. Woch.*, 1904, xxx, 1458.

⁴ Neufeld, F., and Rimpau, W., *Z. Hyg. u. Infektionskrankh.*, 1905, li, 283.

⁵ Wright, A. E., On pharmo-therapy and preventive inoculation applied to pneumonia in the African native, New York, 1915, 78.

⁶ Heist, G. D., Solis-Cohen, S., and Solis-Cohen, M., *J. Immunol.*, 1918, iii, 261.

⁷ Heist, G. D., and Solis-Cohen, S., *J. Immunol.*, 1919, iv, 147.

⁸ Avery, O. T., Chickering, H. T., Cole, R., and Dochez, A. R., Acute lobar pneumonia. Prevention and serum treatment, Monograph of The Rockefeller Institute for Medical Research, No. 7, New York, 1917.

⁹ Winternitz, M. C., and Kline, B. S., *J. Exp. Med.*, 1915, xxi, 320.

¹⁰ Stryker, L. M., *J. Exp. Med.*, 1916, xxiv, 49.

within a few minutes, the blood of an animal having a pneumococemia. This last phenomenon has been ascribed to the agglutinating and bacteriotropic properties of the immune serum and the phagocytic cells of the host rather than to a bactericidal action of the serum.¹¹ Since the reactions here concerned take place within the whole blood, it would, in the light of the reports of Wright and Heist and his coworkers, be reasonable to suppose that the bactericidal property of the whole circulating blood of the immune animal plays an important part in this phenomenon.

EXPERIMENTAL.

Technique of the Whole Blood Test.^{6, 6}—Sets of five or more capillary glass tubes about 1 mm. in diameter are filled respectively, by capillary attraction, to a fixed point with graded dilutions of an 18 hour blood broth culture of pneumococci, the culture being immediately drawn from the tubes by touching the filled ends with sterile gauze. This leaves a coating of culture on the walls of the tubes, the number of bacteria left behind varying with the dilution of the culture. When the tubes have dried in the air for a few minutes they are filled to the fixed point with the blood to be tested by touching them to a drop of blood as it flows from a punctured vein. The tubes are sealed by dipping them into melted paraffin (both ends may be sealed, the loaded end first). They are incubated at 37°C. for any desired length of time and then examined by making films of the contents, staining, and examining with the microscope, or by making cultures in suitable medium. It is expedient to make five or more dilutions of the culture, varying from undiluted culture to a dilution of 1 : 1,000, broth being used as the diluting fluid. Three or more sets of capillary tubes should be prepared for each test in order that a set of dilutions may be examined after different incubation periods, varying from 1 to 96 hours as the test may demand.

For microscopic examination the entire contents of a tube are blown upon a slide and spread thinly over the surface. The films are allowed to dry in the air, fixed in methyl alcohol, and stained with Manson's stain. To avoid overlooking the bacteria, especially when they are not evenly distributed, it is necessary to go over the whole preparation with great care. When diplococci are found in the preparations it indicates that multiplication has occurred, for on control slides made immediately after the tubes are loaded, bacteria are never found, except when undiluted culture has been used to charge the

¹¹ Bull, C. G., *J. Exp. Med.*, 1915, xxii, 457.

tubes, and even here the slides are usually negative. Cultures are made from the tubes by blowing the contents into the condensation fluid of freshly prepared rabbit blood agar slants, and the clot, when whole blood is being tested, is broken up and spread over the surface of the slant. The inoculated tubes are examined after from 24 to 48 hours incubation.

In determining the relative effects of the blood of different animals on the growth of the culture it is essential that the tests should be prepared simultaneously and with the same dilutions of culture. All other conditions of the tests must be the same in each instance, for slight variations will give incorrect results. It is well to run a control set of tubes with normal rabbit blood in order to establish the viability and vigor of the culture.

Any digression from these general directions for technique will be pointed out in the protocols.

The distinctive and important feature of the tests is that the organisms are brought into contact with the undiluted blood or other fluid to be tested.

Experiment 1.—In these tests with pigeon blood the capillaries were charged with dilutions of culture ranging from undiluted culture to a dilution of 1 : 625. Sets of tubes were examined after from 1 to 72 hours incubation. Control tests with normal rabbit blood were made in each instance. Fresh pigeon serum was also used as a control.

Pigeon blood, as compared with rabbit blood, greatly retards the multiplication of pneumococci. Capillaries charged with undiluted culture and filled with rabbit blood are positive on microscopic examination after from $\frac{1}{2}$ to 1 hour incubation, while those filled with pigeon blood do not become positive until after an incubation period of from 6 to 18 hours. Capillaries with a 1 : 625 dilution of culture become positive in rabbit blood after from 5 to 7 hours incubation, while in the tubes filled with pigeon blood organisms cannot be found until after about 72 hours. Tubes with intermediate dilutions of culture become positive after a corresponding incubation period. All the tubes give positive cultures at any time during the test (Table I).

A negative microscopic examination does not necessarily mean that the pneumococci have been killed, but a negative culture is essential to such a conclusion. As far as the rabbit and pigeon are concerned, the rapidity with which the cultures develop may be taken as a fair index of their relative susceptibility to infection by these organisms.

TABLE I.

Pneumococcus Cultures in Fresh Pigeon Blood; Microscopic versus Culture Test.

Dilution of culture with which capillary was charged.	Incubation.	Microscopic examination.	Culture on rabbit blood agar.
	<i>hrs.</i>		
Undiluted.	1	—	+
1 : 5	1	—	+
1 : 25	1	—	+
1 : 125	1	—	+
1 : 625	1	—	+
Undiluted.	6	—	+
1 : 5	6	—	+
1 : 25	6	—	+
1 : 125	6	—	+
1 : 625	6	—	+
Undiluted.	12	+	+
1 : 5	12	—	+
1 : 25	12	—	+
1 : 125	12	—	+
1 : 625	12	—	+
Undiluted.	18	+	+
1 : 5	18	+	+
1 : 25	18	—	+
1 : 125	18	—	+
1 : 625	18	—	+
Undiluted.	42	+	+
1 : 5	42	+	+
1 : 25	42	+	+
1 : 125	42	+	+
1 : 625	42	—	+
Undiluted.	65	+	+
1 : 5	65	+	+
1 : 25	65	+	+
1 : 125	65	+	+
1 : 625	65	+	+

Experiment 2.—Comparative tests were made with the blood of a number of other laboratory animals. The microscopic method was used to determine whether growth had occurred, a positive result meaning that diplococci were found in the stained preparations. Sufficient cultures were made to eliminate the possibility of contaminations. Since the tubes always became positive in the order of the dilutions of culture with which they had been charged, only the results of the two extreme dilutions are given. Table II gives a summary of these tests.

Table II includes only normal individuals of the different species which are arranged in the order of the incubation (latent) period of

TABLE II.

Latent Period of Pneumococcus Cultures in the Whole Blood of Different Animals.

Animal.	Length of time after which cultures are positive on microscopic examination.	
	Capillaries charged with undiluted culture.	Capillaries charged with 1:625 dilution of culture.
	<i>hrs.</i>	<i>hrs.</i>
Rabbit.....	$\frac{1}{2}$ -1	3-5
Mouse.....	$\frac{1}{2}$ -1	4-6
Cat.....	1-2	5-6
Guinea pig.....	1-2	6-7
Sheep.....	2-3	6-7
Man.....	2-3	6-7
Dog.....	3-4	8-12
Hen.....	4-5	12-15
Pigeon.....	6-12	48-72

pneumococci in the respective bloods. It is seen that the rabbit, mouse, and cat come at the top of the list, the pigeon, hen, and dog at the other end, while the guinea pig, sheep, and man occupy intermediate positions. There is no doubt that in a general way this is the order of susceptibility to infection of these species to the pneumococcus, the rabbit and mouse being the most susceptible and the pigeon and hen the most resistant; but the relative positions of those falling in the middle zone are questionable. The cat, for example, should be farther separated from the rabbit and mouse, particularly on intravenous inoculation. Further tests showed that the method is not sensitive enough to classify normal individuals of the same species

with respect to susceptibility to pneumococcus infection. Experiments on vaccinated rabbits showed, however, that in this instance, an immunity reaction which is not made evident by other methods used to demonstrate antibodies^{6, 7} can be detected by this method. The data recorded in Experiment 3 illustrate this point.

Experiment 3.—Each of two rabbits was given intravenously the killed cultures from two blood agar slants of pneumococci. On the 3rd day after the injection the rabbits were bled and the sera tested in the ordinary way for agglutinins and opsonins. Whole blood tests were set up at the time the blood was collected for the serum. The tests were repeated on each succeeding day, corresponding tests being made on normal rabbits as controls. Even on the 3rd day the blood of one of the treated rabbits caused a slight delay in the growth of the culture. On the 4th day the blood of both rabbits caused a definite lengthening of the latent period and this effect rapidly increased on the succeeding days, while agglutinins and opsonins were not demonstrable in the serum, by ordinary methods, until the 6th day, although 50 per cent serum was present in the tests.

As may be inferred from Experiment 3, active immunization of rabbits against pneumococci confers upon their blood the property of retarding the growth of these organisms, the actively immune rabbit conforming to the normal pigeon in this respect. Further experiments showed that passive immunity gives identical results. Guinea pigs and rabbits were used in these experiments.

The foregoing experiments show that, in a general way, the rapidity with which pneumococci multiply in the fresh whole blood of various animals is directly proportional to the susceptibility of these animals to infection by the pneumococcus. Slight differences in susceptibility cannot, at least in every instance, be detected by this method. The cat, for example, is many times more resistant than the rabbit to infection with pneumococci on intravenous inoculation, while the whole blood test would indicate that the cat is only slightly more resistant. It is also shown that natural and artificial immunity—both active and passive—are similar with respect to this test. Finally, the conclusion that the fresh uncoagulated blood of immune animals is highly pneumococcidal^{5, 6, 7} is doubtful, since the mixtures did not become sterile during the course of the experiments and multiplication of the organisms always occurred when incubation was sufficiently prolonged. On the other hand, it cannot be concluded

that no killing occurs; but to prove positively that the blood possesses a pneumococcidal property it would be essential that some of the pipettes, for example those charged with the higher dilutions of culture, should become free of viable organisms. The phenomenon described is, however, closely associated with, and at least roughly parallel to, resistance to infection. It was thought, therefore, that the working out of the mechanism of the reaction would possibly give important information as to the nature of the factors and processes involved in pneumococcus immunity. A large number of experiments have been carried out with this end in view.

Microscopic Study.

A microscopic study was made of cultures of pneumococci at different stages of development in the whole blood of the animals used in the foregoing experiments. Capillary tubes were charged with the culture and loaded with the fresh uncoagulated blood according to the method already described. Sufficient tests were prepared in each instance to permit of frequent examinations. Cultures made in the blood of normal rabbits were used as controls, rabbit blood being an excellent medium for pneumococci. The following points were noted: (1) the kind of clot formed by the different bloods, (2) distribution of diplococci in the medium, (3) chain and clump formation, and (4) phagocytosis.

Clot Formation.—The bloods of different species behave differently with respect to retraction of the clot within the capillary tubes. The rabbit clot retracts both transversely and longitudinally, usually pulling away from the walls of the tube within a short time. This results in a small clot, and a relatively large amount of free serum. The pigeon clot retracts very little in any direction and usually continues to fill the whole tube, and there is only a small amount of free serum. The hen clot usually retracts longitudinally, but less than that of rabbit blood, and pulls away from the walls of the tube to a much less degree than the latter. The other bloods come between these extremes with respect to retraction of the clot. This point will be referred to later, since it apparently plays at least a secondary part in the phenomenon under investigation.

Distribution of the Organisms in the Culture.—Three films were made from each capillary culture, (1) from the free serum, (2) from the serum adhering to the clot, and (3) from the clot. The serum was removed from the small culture tubes by means of a very fine capillary. The clot was blown upon a slide and moved over the surface in order to make a film of the serum adhering to the clot. The clot was then dried on filter paper and a preparation made of the dried clot. These preparations were made at all stages of the development of the cultures and it was found that in the earlier stages the pneumococci were multiplying actively in the serum, fewer organisms were in the serum adhering to the clot, and the clot itself was not invaded until the cultures were well developed. This was particularly true of the capillaries containing immune blood—pigeon, hen, or immune rabbit blood. The clot of normal rabbit blood was invaded earlier in the development of the culture. This distribution might have been predicted because (1) the seeding organisms are at the periphery of the clot, and (2), as has been stated, pneumococci grow readily in the most potent immune serum.

Chain and Clump Formation.—Just as in immune serum, pneumococci grow in chains and clumps in immune blood, the growth in this instance being largely in the serum after coagulation has taken place. In pigeon blood there is only a slight clumping but long chains are formed, while in hen blood large clumps develop and also long chains. Chains and clumps are formed in immune rabbit blood, the size of the clumps and the length of the chains varying with the degree of immunity and the incubation time. The first indication of the development in rabbits of the immunity response to an inoculation of the organisms is chain formation, and the second, clump formation. In highly immune blood, the growth is restricted to chains and clumps until the culture approaches maturity when the growth may become diffuse (uniformly distributed diplococci) within a short time. The chain and clump phases of the cultures remind one of local infections and the diffuse phase of general infections. Apparently growth does not become diffuse until the antibodies of the blood have been exhausted, or until the organisms have become less susceptible to the antagonistic influence of the blood.¹² The chain and clump phenom-

¹² Bull, C. G., *J. Exp. Med.*, 1916, xxiv, 7.

ena occur in pigeon blood, hen blood, in the blood of an immune rabbit or any other immunized animal, and to a less degree in normal dog blood. They do not occur in normal rabbit, mouse, or cat blood. This fact must be kept in mind when making microscopic examination of cultures in immune blood; otherwise, even massive clumps may be overlooked and the specimen pronounced negative.

Phagocytosis.—Phagocytosis of the pneumococci was first observed in preparations made from cultures in hen blood. For this purpose the cultures should be examined after from 4 to 5 hours incubation. Preparations from the serum adhering to the clot contain the greatest number of phagocytizing cells (polymorphonuclear leucocytes). Phagocytosis occurs in any immune blood. In order to demonstrate this appearance to advantage in highly immune bloods (pigeon, immune rabbit) it was necessary to charge the tubes with concentrated suspensions of the organisms. This was effected by centrifugalization and resuspension of broth cultures. A tenfold concentration gave good results. When more dilute cultures are used, the number of phagocytizing cells is small and satisfactory observations cannot be made.

The tentative hypothesis which we based on the above observations was that the development of the culture to the point of being positive on microscopic examination was retarded by chain and clump formation and possibly by phagocytosis. When the organisms are held tightly in a few clumps, instead of being uniformly distributed through the medium, there is less probability of observation with the microscope. Thus, of two preparations containing the same number of organisms, one clumps and the other evenly distributed diplococci, the first, barring accident, could easily be found negative and the second positive. Moreover, multiplication is probably actually retarded because of reduced nutrition or an accumulation of inhibitory substances in the interior of the clump. In regard to phagocytosis the notion was that either a certain number of the pneumococci were killed by the phagocytes or multiplication within the phagocytes was held in check for a time, thus retarding the development of the culture. Further work was planned to determine the part played by the leucocytes in the reaction.

Phagocytosis and Retardation of Growth.

If phagocytosis is essential in the retardation of the cultures in immune blood, it is evident that the phenomenon depends on two agents; *viz.*, opsonins and leucocytes. The reaction should not occur in the absence of either factor, and one of the factors being constant the retardation should be directly proportional, within certain limits, to the quantity of the other factor present. This conception of the problem guided the experiments recorded below.

Defibrinated Blood.—It is stated by Heist and his associates⁶ that defibrinated immune blood (chicken) is as good a medium for pneumococci as defibrinated normal rabbit blood. We studied this point since it appeared that phagocytosis does not play an essential part in the retardation of the growth of the culture, since the leucocytes are present in defibrinated blood, presumably, as in whole blood. Immune rabbit blood was used in this study.

Experiment 4.—Capillary tubes were prepared as usual with (1) whole blood, (2) defibrinated blood, and (3) serum. The tests were set up simultaneously, the capillaries having been charged with the same dilutions of culture. The defibrinated blood and serum tests were prepared a few minutes after the blood had been collected by cardiac puncture. The defibrination was effected by shaking the blood with glass beads and the serum was collected by centrifugalizing a portion of the blood immediately after coagulation. The whole blood was collected from the ear vein.

By examining the cultures at short intervals it was found that the pneumococci multiply more rapidly in the defibrinated blood than in the whole blood, but growth in the serum is still more rapid than in the defibrinated blood (Table III).

Leucocytes of Defibrinated Blood.—Whole and defibrinated blood from the same animal were studied in regard to the number, type, and condition of the leucocytes present, total and differential counts being made. It was found that defibrination removes from one-half to three-fourths of the total leucocytes, and a large percentage of those remaining in the defibrinated blood are lymphocytes. In one instance the following observations were made: whole blood, total count 12,515, polynuclear cells 7,460, lymphocytes 4,880, mononuclear leucocytes 175; defibrinated blood, total count 3,900, polynuclear cells 835, lymphocytes 2,905, mononuclear leucocytes 160. In other instances the total count was not reduced to such a low figure but the

number of polynuclear cells remaining in the defibrinated blood was always relatively very low. It was further noted that the process of defibrination visibly injured a large percentage of the polynuclear cells.

In these experiments there was, at least roughly, a mathematical relation between the number of polynuclear cells present and the rate

TABLE III.

Pneumococcus Cultures in the Whole Blood, Defibrinated Blood, and Serum of an Immunized Rabbit and in Normal Blood.

Dilution of culture with which capillary was charged.	Incubation. <i>hrs.</i>	Microscopic examination.			
		Whole blood.	Defibrinated blood.	Serum.	Normal rabbit blood.
Undiluted.	4	—	+	+	+
1 : 5	4	—	—	+	+
1 : 25	4	—	—	—	+
1 : 125	4	—	—	—	+
1 : 625	4	—	—	—	—
Undiluted.	8	+	+	+	+
1 : 5	8	—	+	+	+
1 : 25	8	—	+	+	+
1 : 125	8	—	—	+	+
1 : 625	8	—	—	—	+
Undiluted.	12	+	+	+	
1 : 5	12	+	+	+	
1 : 25	12	+	+	+	
1 : 125	12	—	+	+	
1 : 625	12	—	—	+	

of multiplication of the pneumococci. The points determined also eliminate the red cells as a decisive factor in the reaction.

Relation between the Number of Cells Present and Retardation of Growth.—The experiments given here corroborate the conclusion drawn from the study of the defibrinated blood; *i.e.*, the latent period of the culture is directly proportional to the number of leucocytes present.

Experiment 5.—Blood was collected from immune animals (immunized rabbits and hens) in oiled glassware, quickly centrifugalized at high speed, and the three layers (plasma, leucocytic cream, and cells) were separated, remixed in different proportions, and the mixtures run into culture-charged capillary tubes before co-

TABLE IV.

Pneumococcus Cultures in Hen Blood, Serum, Plasma, and Equal Parts of Plasma and Leucocytic Cream.

Dilution of culture with which capillary was charged.	Incubation.	Microscopic examination.			
		Whole blood.	Serum.	Plasma.	Equal parts of plasma and leucocytic cream.
	<i>hrs.</i>				
Undiluted.	4	+	+	+	+
1 : 5	4	+	+	+	—
1 : 25	4	—	+	+	—
1 : 125	4	—	—	—	—
1 : 625	4	—	—	—	—
Undiluted.	8	+	+	+	+
1 : 5	8	+	+	+	+
1 : 25	8	+	+	+	—
1 : 125	8	+	+	+	—
1 : 625	8	—	+	+	—
Undiluted.	12	+			+
1 : 5	12	+			+
1 : 25	12	+			+
1 : 125	12	+			—
1 : 625	12	+			—
Undiluted.	24				+
1 : 5	24				+
1 : 25	24				+
1 : 125	24				+
1 : 625	24				+

agulation occurred. Washed guinea pig leucocytes and plasma or serum mixtures were also tested.

The following observations were made (Tables IV and V). (1) *Pneumococci* multiply rapidly in both the serum and coagulated plasma of immune animals, in some instances growth being more rapid in the plasma than in the serum. (2) Plasma and cells mixed in equal amounts inhibit growth to the same degree as the original whole blood. (3) A mixture of equal quantities of plasma and leucocytic cream delays the development of the culture more than the original whole blood.

TABLE V.

Pneumococcus Cultures in Hen Blood, Plasma, Sedimented Cells, and Ground Sedimented Cells.

Dilution of culture with which capillary was charged.	Incubation. <i>hrs.</i>	Microscopic examination.			
		Whole blood.*	Plasma.	Sedimented cells.†	Ground sedi- mented cells.
1 : 10	11	+	+	—	+
1 : 100	11	+	+	—	+
1 : 1,000	11	—	+	—	+
1 : 5,000	11	—	+	—	+
1 : 10,000	11	—	—	—	—
1 : 10	28	+	+	—	+
1 : 100	28	+	+	—	+
1 : 1,000	28	+	+	—	+
1 : 5,000	28	+	+	—	+
1 : 10,000	28	—	+	—	+
1 : 10	58	+		+	
1 : 100	58	+		—	
1 : 1,000	58	+		—	
1 : 5,000	58	+		—	
1 : 10,000	58	+		—	
1 : 10	72			+	
1 : 100	72			+	
1 : 1,000	72			+	
1 : 5,000	72			—	
1 : 10,000	72			—	
1 : 10	96			+	
1 : 100	96			+	
1 : 1,000	96			+	
1 : 5,000	96			+	
1 : 10,000	96			+	

* The hen was given 10 cc. of antipneumococcus serum 30 minutes before the blood was collected.

† Cultures of the sedimented cells on rabbit blood agar were positive throughout the experiment.

(4) Plasma plus washed leucocytes delays growth. (5) Immune serum and leucocytes do not retard growth to the same degree as plasma and leucocytes. (6) The compact cells¹³ of a highly immune blood produce a prolonged latent period in the cultures which do not become positive on microscopic examination until after from 48 to 72 hours. (7) If the cells, as described under (6), are thoroughly ground with a mortar and pestle in fine, sterile sand, the pneumococci multiply as rapidly as in the serum or plasma. This is also the case when leucocytic cream or leucocytes are used in making the mixtures.

Here again it is indicated that the presence of leucocytes is essential to the retarding property of the blood, and since a mechanical destruction of the phagocytes, nothing being removed or added, robs the plasma-cell mixtures of their retarding property, it seems to be established that the act of phagocytosis plays a decisive part in the reaction. It remains to be explained, however, why a mixture of leucocytes and immune serum does not retard the development of the culture to the same extent as the plasma of the animal plus an equal number of leucocytes. A mechanical factor is involved here. In the serum-leucocyte mixtures the leucocytes quickly settle to the bottom of the capillary, leaving a layer of serum in which the pneumococci rapidly multiply. It should be stated here that when chicken plasma is prepared as described above the clot does not retract in the tubes but continues to occupy the whole tube, thus bringing the leucocytes into direct contact with the pneumococci which have been deposited on the walls.

Concentration of Antibodies and Retardation of Growth.

Experiment 6.—A series of guinea pigs was given intravenous injections of varying quantities of antipneumococcus horse serum, the amount of serum injected ranging from 1 to 5 cc. 2 hours later capillary cultures were made of the whole blood of the guinea pigs. A normal guinea pig was included in the series. A set of the cultures was examined every 2 hours.

The latent period of the cultures was directly proportional to the quantity of serum injected, the cultures in the blood of the guinea pig which received 1 cc. of serum having a longer incubation period than those in normal blood, while the cultures made in the blood of the animal which received 5 cc. of serum were the last to develop (Table VI).

¹³ The cells were sedimented by centrifugalization, but sufficient serum to opsonize the bacteria still adhered to the cells.

TABLE VI.

*Pneumococcus Cultures in the Blood of Passively Immunized Guinea Pigs.**

Dilution of culture with which capillary was charged.	Incubation. <i>hrs.</i>	Microscopic examination.		
		Guinea Pig A (1 cc. of immune serum).	Guinea Pig B (5 cc. of immune serum).	Guinea Pig C (normal control).
Undiluted.	4	+	—	+
1 : 5	4	—	—	+
1 : 25	4	—	—	+
1 : 125	4	—	—	—
1 : 625	4	—	—	—
Undiluted.	8	+	+	+
1 : 5	8	+	—	+
1 : 25	8	+	—	+
1 : 125	8	+	—	+
1 : 625	8	—	—	+

* The antipneumococcus serum was given intravenously 2 hours before the cultures were made.

DISCUSSION.

The observations outlined above indicate that the whole blood of immune animals merely retards the multiplication of pneumococci instead of killing them, as is claimed by Wright and Heist and his coworkers. It is clearly demonstrated that failure to develop within 24 hours to the point of being positive on microscopic examination does not prove that the organisms with which the cultures were seeded have been killed. We have observed the development of cultures after a latent period of 72 hours. The authors referred to above ended their experiments after an incubation period of 24 hours. Should the organisms never multiply in the original medium, it could not be concluded that they had been killed, unless the cultures were proved to be sterile by failure of growth when transferred to a highly favorable medium. Even in this instance, particularly after a prolonged original incubation period, the possibility of involution would not be eliminated. It is claimed by Heist and his collaborators that a negative microscopic examination after 24 hours incubation invariably means pneumococidal effect. The sterility test employed, however, was that of blowing the contents of the capillary tubes into broth.

The small number of organisms thus transferred was probably unable to overcome the resistance of the relatively large volume of liquid medium. In our experiments fresh rabbit blood agar tubes were substituted for the broth.

In every instance so far observed the degree of retardation of growth of pneumococci exerted by the whole blood corresponded to the degree of resistance of the corresponding animal to infection by these organisms. The converse, *i.e.* the absence of power in the blood to retard growth, is probably not always a true index of the susceptibility of the animal yielding it to infection. This seems to be true in the case of the cat, particularly on intravenous inoculation.

The whole blood reaction, as pointed out by Heist and his co-workers,⁶ makes it possible to detect immunity responses which are not made evident by other methods. Only rabbits were used in the experiments on this point and our conclusions are correspondingly limited. We were unable to detect differences among untreated individuals of the same species. Hence it is improbable that the reaction will enable the detection of pneumonia susceptibility among human individuals, as the Schick test does for diphtheria.

The mechanism of the reaction is of particular interest, especially in connection with the light that its explanation may shed upon the nature of the factors and processes of pneumococcus immunity. Wright⁵ designated the phenomenon as the "phagocyto-bactericidal power" of the blood, believing that the phagocytes were concerned in the reaction since plasma did not manifest it; but since defibrinated blood was inactive in this respect, he concluded that the chemical changes associated with the process of coagulation of the blood were essential factors in killing the pneumococci. The present work indicates that opsonization and phagocytosis are the essential agents, since the pneumococcal reaction does not occur in the absence of either factor. It was shown, moreover, that with either factor constant the reaction varied quantitatively with the quantity of the second factor. It is conceivable that this relation will not be maintained when immune serum exceeds the concentration necessary for complete opsonization of all the organisms, the phagocytic capacity remaining constant. On the other hand, complete opsonization being maintained, there should be no limit to the number of organisms which

an increasing number of phagocytes could hold in check. This relation is similar to that which exists between immune serum and pneumococci in mouse protection experiments. In the latter instance, a certain quantity of antipneumococcus serum (0.2 cc.) protects against a maximum number (0.1 cc. of culture) of organisms, but when the number of organisms is increased, 0.2 cc. or larger quantities of the serum do not protect. This fact indicates that some essential factor (phagocytic capacity?) which is not increased has reached the limit of its power and the reaction fails. To carry the analogy further, the functioning time of the phagocytes in the capillary cultures is limited, and at the expiration of the period of phagocytic activity the reaction ceases. The pneumococci are now free to multiply regardless of the fact that immune bodies are still present. In the mouse protection tests a concentration of immune serum sufficient for complete opsonization continues for a limited time only. If the infection is so heavy that the phagocytes, functioning to the limit of their capacity, cannot destroy all the pneumococci during the opsonization period, some of the organisms escape destruction and are free to grow as soon as the opsonizing factor is eliminated. It has been shown that if the immune serum is kept at a sufficient concentration in the tissues of the infected animals by repeated injections, thus giving the phagocytes a longer working period, all the organisms are destroyed and the animal recovers.¹⁴ This effect is not subject to duplication *in vitro* since the phagocytes which cease functioning because of death cannot be replaced.

It has not been definitely determined whether the retardation of growth of the culture is due merely to an inhibition of multiplication or to an actual destruction of a portion of the organisms, thus prolonging the incubation period. Indications are, however, that the action is one of inhibition only, since none of the cultures became sterile during the course of the experiment. It will be necessary to do further work before the matter is definitely cleared up.

¹⁴ Bull, C. G., *J. Exp. Med.*, 1915, xxii, 466.

SUMMARY.

1. It has been shown that the whole uncoagulated blood of immune animals is not as highly pneumococcal *in vitro*, as has been claimed by others.

2. Cultures of pneumococci in the fresh whole blood of immune animals, as compared with cultures in the blood of susceptible animals, show a greatly prolonged latent period, and, in a general way, the relative lengths of the latent periods of the cultures correspond to the relative resistances of the animals to infection by these organisms.

3. The blood of animals artificially immunized, both actively and passively, retards the growth of pneumococci in the same manner as the blood of naturally immune animals.

4. Microscopic examination of cultures of pneumococci in immune blood reveals chain formation, growth in clumps, and phagocytosis of the organisms by the polynuclear cells. It also shows that growth occurs first in the free serum, the clot being invaded later.

5. The retardation of multiplication depends on two factors, opsonization of the pneumococci by the immune serum and phagocytosis of the organisms by the polynuclear cells; growth readily occurs when either agent is absent.

6. Pneumococci multiply in defibrinated immune blood because few phagocytes are present after defibrination.

7. Pneumococci grow in the most potent immune blood after mechanical destruction of the white cells.

8. It has not been shown that immune blood does not kill a certain number of the pneumococci with which it is inoculated, but the tentative conclusion has been arrived at that no killing occurs since none of the tests became sterile during the course of our experiments.

EXPERIMENTS ON THE PRODUCTION OF SPECIFIC ANTISERA FOR INFECTIONS OF UNKNOWN CAUSE.

III. THE EFFECTS OF A SERUM PRECIPITIN ON ANIMALS OF THE SPECIES FURNISHING THE PRECIPITINOGEN.

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It has been shown previously that specific antisera for infections of which the exciting agent is unknown can be produced in some instances by the use of infected tissue itself as antigen; and that when such sera have been detoxified they can be used successfully for therapeutic purposes.¹ The toxicity is due to the presence of antibodies elicited by the tissue component of the antigen, which, needless to say, are highly injurious to animals of the species furnishing said antigen. It is easy to remove the more obvious of these injurious elements—hemolysins and hemagglutinins—by their selective absorption with red corpuscles, as was done in our type experiments. The precipitins we then encountered were weak and without recognizable action on the animal organism. Whether strong precipitins can cause damage has remained for determination, as has the point of how such damage could be avoided. The problem is not without practical significance in its relation to the utilization of the serum of infected human individuals as antigen.

The study of anaphylaxis has led to the development of a large and complex literature on the effects of the union of precipitin and precipitinogen upon an animal organism to which both are foreign; and a number of papers have been published on the results of injecting precipitinogen into animals which have developed a circulating precipitin. But there exist few observations, and these more or less casual, regarding the effects of a precipitin upon animals against

¹ Rous, P., Robertson, O. H., and Oliver, J., *J. Exp. Med.*, 1919, xxix, 283.

whose serum it is specifically effective. This alone concerns us here. Uhlenhuth and Haendel² state in a foot-note to other matters that guinea pigs show severe, anaphylaxis-like symptoms after the intraperitoneal injection of 0.5 to 1 cc. of an anti-guinea-pig rabbit serum with a precipitin titer of 1 to 20,000. The same serum in amounts of 0.75 to 2 cc. caused death. They give a few protocols suggesting tolerance to a second injection. Doerr and Moldovan³ made closer observations. They state that the precipitating anti-guinea-pig rabbit serum which they employed had *in vitro* a slight hemolytic activity for guinea pig cells, no more than that of normal rabbit serum, yet 2 cc. intravenously killed guinea pigs within 2 hours, while 1 cc. gave rise to severe symptoms, and 0.5 cc. to dyspnea and slight symptoms. Following an intraperitoneal injection there was heightened resistance to a second injection 24 hours later. The experiments cited are few; and the authors make no mention of the presence or absence of hemagglutinins, nor, it seems, did they look for evidence of *in vitro* hemolysis. These are important points for the interpretation of their work, as will be shown.

Removal of Hemolysins and Hemagglutinins.

It is well known that immunization with blood serum as antigen leads to the development not only of precipitins but of hemolysins and hemagglutinins, even though care has been taken to render the serum free of formed elements. In our first experiments along the lines of the work just described it became evident that these antibodies were seriously to be reckoned with. Hemolysis was never noteworthy on *in vitro* tests, being practically absent when guinea pig complement was employed, and in this regard our sera corresponded with the serum of Doerr and Moldovan. Yet these same sera, inactivated, gave rise *intra vasam* to a profuse breaking down of red cells, as evidenced by hemoglobinuria, hemoglobinemia, extreme anemia, spodogenous spleen, and free blood pigment in the fluid of the body cavities. Some fatalities were manifestly attributable to this cell destruction combined with hemagglutination. The latter phenomenon was always pronounced both *in vitro* and *in vivo*. That death can be due to it alone is well recognized. The blood of the animals sometimes showed an almost massive clumping. In view of all these findings the fact that the precipitating sera gave rise, as they did, to the more or less sudden death of guinea pigs was not surprising.

² Uhlenhuth and Haendel, *Z. Immunitätsforsch., Orig.*, 1909-10, iv, 761.

³ Doerr, R., and Moldovan, J., *Z. Immunitätsforsch., Orig.*, 1910, vii, 223.

In order to free the sera of hemolysins and hemagglutinins resort was now had to selective absorption with guinea pig red cells. Our technique for this has been described in a previous paper.¹ Care was taken to keep the serum sterile and to free it of all possible stroma fragments by prolonged centrifugation prior to injection. Also, it was overabsorbed, that is, exposed to far more guinea pig red cells than were enough to remove the demonstrable antibodies for these elements. Our experience,¹ like that of others, has been that the strong hemolytic and hemagglutinative serum resulting from immunization with red cells is completely deprived of toxicity when thus treated. Such was far from being the case with the precipitating serum now in question. After absorption it retained the major part of its toxicity, giving rise to sudden death almost as often, in almost the same dose, and with the same symptoms as when untreated save for inactivation. But there was the difference that all lesions referable to hemolysis and hemagglutination were now lacking. Thus a number of late fatalities were avoided. Manifestly, from these results, Doerr and Moldovan were correct in supposing that the toxicity of their serum was due to another element than hemolysis.

Removal of the Precipitin Does Not Remove Toxicity.

What is this other element? The guinea pig cells used in the absorptions were freed of serum by careful and repeated washing in "gelatin-Locke's" solution, which keeps these usually fragile cells intact. The rabbit serum repeatedly incubated with several successive portions of them remained clear and almost free from hemoglobin. Friedemann⁴ has shown that mixtures of red cells and hemolysin in the presence of complement may yield a toxic body before any hemolysis occurs. But this does not happen when complement is absent, as was regularly the case in our work. The possible influence of traces of the washing solution can be ruled out on the basis of previous experience. It seemed likely that the precipitin content of the serum, which was not lessened by repeated absorptions, constituted the toxic element. And in line with this idea, though not necessarily evidence for it, was the fact that the toxicity of different serum specimens

⁴ Friedemann, U., *Z. Immunitätsforsch., Orig.*, 1909, ii, 591.

varied in general with their precipitin titer. Attempts were made, therefore, to detoxify the sera by the removal of their precipitin content through specific precipitation. The precipitin was readily removed. But to our great surprise the sera remained as toxic as before.

Specimen Experiments.

Experiment 1.—Two rabbits which had received five intraperitoneal injections of guinea pig serum at intervals of 6 days were bled to death from the heart 10 days after the last, and the serum was at once pooled, inactivated, and tested for hemolysin and hemagglutinin. The undiluted serum caused a faint trace of hemolysis when incubated for 2 hours with equal parts of a 5 per cent suspension of guinea pig red cells and a 1 in 10 dilution of fresh guinea pig serum. In such mixtures agglutination was massive with quarter strength serum and was faintly seen with a 1 in 32 dilution of it. The injection of 2 cc. of the serum into the ear vein of a 200 gm. guinea pig⁵ was followed in a few minutes by sneezing, restlessness, severe dyspnea, and complete prostration, with slow recovery during the next 12 hours.⁶ The urine for some hours after the injection contained much hemoglobin.

50 cc. of the inactivated serum was now incubated under aseptic conditions with four successive portions of guinea pig red cells, twice washed in a large excess of gelatin-Locke's solution. The portions consisted of 7.5, 7.5, 9, and 6 cc. of packed cells respectively, and the period of incubation ranged from $\frac{3}{4}$ to 3 hours. No agglutination was observable in the last two serum-cell mixtures, and *in vitro* tests showed the complete absence of hemolysin. The absorbed serum, when injected into two guinea pigs of 225 and 215 gm. weight, in amounts of 2 and 1.35 cc. respectively, gave rise to exactly the same symptoms as the unabsorbed, though they were somewhat less severe. The urines of both animals remained free from hemoglobin.

The precipitin titer of the serum was now taken in mixtures of a constant amount of antibody with decreasing antigen, so as to avoid solution of the precipitate in an excess of the latter. By the use of a blood-counting pipette, previously standardized with mercury, as a measuring chamber, small amounts of the undiluted sera were mixed as such. Precipitation occurred in mixtures up

⁵ For the technique of such injections see Rous, P., *J. Exp. Med.*, 1918, xxvii, 459. The operation is rendered more simple and certain if the ear is fixed on a ground glass platform, instead of the opaque one previously described, and transillumination is employed.

⁶ 3 to 4 cc. of normal rabbit serum can be injected into the circulation of a 200 to 250 gm. guinea pig without the production of symptoms (see Friedberger, E., *Med. Klin.*, 1910, vi, 510).

to and including that containing 5,120 parts of rabbit serum to 1 from the guinea pig, and in 20,000 to 1 when the antigen was diluted to a constant bulk with salt solution after the usual method. The greatest precipitate, coarsely floccular, was seen at approximately 80 to 1 of the whole sera. Accordingly the bulk of the remaining rabbit serum was mixed with sterile guinea pig serum in this proportion, incubated 2 hours, left in the cold over night, and centrifuged until free from the several cubic centimeters of precipitate. It now failed to cause any clouding in mixtures with guinea pig serum above 16 to 1, yielded a slight cloud at 8 to 1, a dubious trace at 4 to 1, and none at 1 to 1. Yet this same serum, injected intravenously into three guinea pigs of 225, 205, and 225 gm. in amounts of 2.2, 1.45, and 1.35 cc. respectively, killed the first animal in 3 hours and 8 minutes, the second in 5½ hours, and in the third gave rise to a moderate "shock" with symptoms resembling those of anaphylaxis.

The results suggest that the serum was rendered, if anything, more toxic by the repeated absorptions.

It is known that a slight, slow precipitation takes place in pooled precipitin sera from different individuals of the same species; and Friedberger⁷ has shown that precipitating mixtures will, in the presence of complement, yield a toxic product *in vitro*. To rule out this possible factor in the results, our tests were repeated with individual rabbit serum; and when several sera were to be pooled they were often subjected beforehand to a separate inactivation. In neither case was any difference noted in the results. In the experiment which now follows the sera were inactivated immediately after pooling, and subjected to precipitation with guinea pig serum prior to the absorptions with red cells.

Experiment 2.—The sera of four precipitin rabbits were pooled, inactivated, the precipitin titer was taken, whole guinea pig serum being used, and on the basis of the findings most of the pooled serum was submitted to an optimum precipitation. This was all done on the same day, as rapidly as possible. The optimum precipitation occurred in a 90 to 1 mixture with undiluted guinea pig serum, but clouding was noted in mixtures up to and including 5,120 to 1. The untreated serum injected intravenously into two guinea pigs of 325 and 400 gm., in amounts of 1.65 and 1.8 cc. respectively, killed the first mentioned animal within ¾ hour and caused great, though brief, prostration of the second. 1.85 cc. of the treated serum killed a 275 gm. guinea pig in 1 hour, and 2 cc. caused moderate symptoms in a guinea pig of 375 gm. All four animals had hemoglobinuria.

⁷ Friedberger, E., *Z. Immunitätsforsch., Orig.*, 1909–10, iv, 636.

Both the treated and untreated sera were now submitted to five successive absorptions with twice washed red cells in the proportion of 23 cc. of serum to 2.3, 2.3, 2.3, 4.05, and 3.8 cc. of packed red cells. The contact periods ranged from 1 to 2 hours. Agglutination, which in the first mixture was well marked, diminished to a trace in the last one—an exactly similar trace for both the “precipitated” and untreated serum, as *in vitro* tests showed. No hemolysin could be found in the test-tube, with guinea pig complement. 1 part of salt solution was now added to 90 of the unprecipitated specimen, and comparative intravenous injections were carried out with it and with the precipitated serum (Table I).

TABLE I.

Serum absorbed only.			Serum absorbed and precipitated.		
Weight of animal.	Amount injected.	Result.	Weight of animal.	Amount injected.	Result.
gm.	cc.		gm.	cc.	
275	2.1	Died in 1 hr., 51 min.	250	2.1	No symptoms.
275	2.0	“ “ 1 “ 5 “	275	1.8	“ “
			275	2.2	Very severe shock.

Tests showed that the absorbed and precipitated serum still contained enough precipitin to cause clouding in mixtures up to and including 320 to 1 with whole guinea pig serum. An optimum was found at 20 to 1 and the serum submitted to a new precipitation in this proportion. Thereafter it still gave a moderate flocculation with an equal amount of guinea pig serum, but no clouding in mixtures above 10 to 1, or 16 to 1 when the precipitinogen was diluted with salt solution. Comparative tests *in vivo* were again made. 1 part of salt solution was added to 20 parts of the unprecipitated serum prior to the injections (Table II).

TABLE II.

Serum absorbed only.			Serum absorbed and precipitated.		
Weight of animal.	Amount injected.	Result.	Weight of animal.	Amount injected.	Result.
gm.	cc.		gm.	cc.	
375	2.0	Moderate shock.			
300	1.8	Severe “			
300	2.15	Died in 11 min.	300	2.2	No symptoms.
275	2.2	Severe shock.	250	2.2	Very severe shock.

At every stage in the treatment of the serum, cultures on agar and in bouillon were taken. These remained uniformly sterile.

In this instance, in contrast with Experiment 1, the attempts to detoxify the serum seemed to have some degree of success, and we were encouraged to further trials. These will not be detailed. They showed that individual differences in the test animals were mainly accountable for the wide variations in the results. Some animals were practically unaffected by the serum that killed others; and no matter how thoroughly the serum was exhausted with red cells and freed of precipitin it remained highly injurious. This was true even when it was given locally. For when injected subcutaneously or into the skeletal muscle it produced a severe lesion. But before describing this its general effects will be taken up.

Effects of the Serum Deprived of Precipitin.

In guinea pigs reacting to an intravenous injection of the exhausted and precipitated—or, for that matter, unprecipitated—serum there is a latent period of from 3 to 10 minutes during which the behavior is normal. Then the animal becomes restless, running about, scratching itself, perhaps sneezing, springing into the air, or twitching. The hair roughens, the urine and feces are usually voided, and an inspiratory dyspnea rapidly appears, accompanied in severe instances by cyanosis and complete prostration, followed by death in a few minutes or hours. Occasionally convulsions precede the fatal issue. Often there is only a paresis of the hind legs, or the animal is now prostrate, now on its feet again, and in these instances of milder symptoms recovery may be very rapid. More often, while recovering, the guinea pig sits crouched, cold, and with staring coat for some hours. When handled it is passive and weak. But by the next day recovery seems complete, and further observation proves that it indeed is so.

The animals that succumb show little on gross examination. The lungs may be distended, and the blood fail more or less markedly to clot, as in anaphylaxis; but these are by no means constant findings. There may be fresh petechiæ in the lungs and intestinal mucosa, as so often after violent death of any sort. This is all that is found if the animal has died within a few minutes of the injection. When it has survived for some hours the liver is always greatly congested, and in it there may be observed microscopically the only lesion that is

present with any regularity; namely, an acute central congestion of the lobuli, often with small hemorrhages, and, if death be deferred long enough, areas of necrosis as a result of the latter. There are no gross hemorrhages in the hepatic tissue. It would seem that the lesion might be secondary to an acute stasis in the heart or lungs, were not an abnormal distension of the right side of the heart entirely lacking at autopsy. In animals surviving several hours polymorphonuclear leucocytes may collect in considerable numbers in the pulmonary capillaries, but in those dying early this feature is absent. The blood shows no hemagglutination and its serum no trace of hemolysis.

The local lesion that follows a subcutaneous injection of 0.5 to 2 cc. of the serum, treated or untreated, is an edema, widespread about the point of injection, with numerous capillary hemorrhages and small, scattered foci of acute inflammation, which, however, do not go on to purulence. The injury may extend into the muscle. A broad, edematous, red-purple patch beneath the skin, with yellowish, ill defined, little, opaque patches and points scattered here and there throughout it, is characteristic. The acute inflammation is at its height at the end of 48 hours, but resolution occurs rapidly thereafter. Cultures remain sterile throughout. Necrosis such as the serum of cattle causes in guinea pigs⁶ is never noted. The ordinary hemolytic and hemagglutinative serum obtained by the repeated injection of washed guinea pig corpuscles into rabbits, causes in guinea pigs a lesion somewhat similar in the gross to the one with which we are concerned; but there is this important difference, that the extravasated red cells which render the patch purple are for the most part hemolyzed so that few are seen on section, and those few are agglutinated into clumps. Furthermore, we have found, as already stated, that absorption with red cells rids such a serum of its ability to cause local lesions other than such slight ones as normal rabbit serum may mechanically produce.

⁶ Uhlenhuth and Haendel, *Z. Immunitätsforsch., Orig.*, 1909, iii, 284.

Parallel Experiments on the Dog.

The work was now repeated on dogs, since for practical purposes it was important to learn whether our findings were peculiar to guinea pigs. The serum employed came from a number of rabbits repeatedly injected with dog serum. The precipitin titer was not so high as in the case of anti-guinea-pig serum, and hemolysins and agglutinins were no stronger, yet per kilo of animal it proved nearly as toxic. The toxicity was unimpaired by absorption with four portions of red cells followed by a single, unusually successful precipitation which removed practically all the precipitin. The intravenous injection of only 2.7 cc. per kilo of the sterile, exhausted and precipitated, or unprecipitated, serum regularly caused marked symptoms in dogs, and resulted once in death after 18 hours. A latent period of 5 to 10 minutes always followed the injection, and then the animal showed sudden signs of weakness, staggering and lying down, or standing unsteadily with drooping head. Vomiting and defecation took place, and soon there was complete prostration, the dog lying on its side in a semiconscious condition. The respiration was not disturbed, but there was an enormous drop in blood pressure, so that the pulse could no longer be felt and sometimes the heart beat was scarcely palpable. In most cases prostration did not endure more than 1 hour, after which recovery was rapid and complete. The animal which succumbed had received absorbed but unprecipitated serum. The single lesion found was similar to that observed in guinea pigs but more pronounced, being a destructive hemorrhagic congestion of the central half of the liver lobules. The blood in the large vessels, which failed to clot, showed no trace of hemagglutination, and the right side of the heart was not greatly distended. The hemorrhages into the liver substance were far more numerous and pronounced than in guinea pigs and this was the case as well with the lesions resulting from local injection in dogs, which otherwise did not differ from those in guinea pigs, save that edema was sometimes less marked. The numerous fine capillary hemorrhages in the edematous tissue at the site of injection often coalesced to give the appearance of a gross extravasate. Normal rabbit serum, old and new, in the amounts here dealt with, failed to cause any local or general disturbances.

Relation of the Phenomena to Anaphylaxis.

The "shock" produced by the serum will be seen from our description to resemble strikingly in both guinea pigs and dogs that called anaphylactic; but on close analysis points of difference declare themselves. At autopsy the lungs of guinea pigs may not be found distended, though they often are so; and the blood may be clotted. The latent period after an intravenous injection is much longer in dogs than that preceding the anaphylactic paroxysm. More important is the fact that desensitization cannot be effected either by small, graduated injections or by one that results in shock. We have given especial attention to this point, since Doerr and Moldovan present a few protocols which seem to indicate that they succeeded in desensitizing with their precipitating serum, and if this were the case it would offer a way to the safe, therapeutic utilization of our own. But their results must be referable to individual animal variation such as has already been mentioned, for our many tests have definitely shown that even after an injection of absorbed, or absorbed and precipitated, serum which calls forth a severe reaction, there may be no tolerance whatever to a second dose, whether it be given into the blood stream or locally. This is true in dogs as well as in guinea pigs. For example, a dog weighing 4 kilos was given 11.6 cc. of exhausted and precipitated serum into an ear vein. There resulted severe "shock," but with rapid and apparently complete recovery. 4 days later another and similar injection was given, and this called forth exactly the same severe but transient reaction. The animal was killed 6 days after the second injection, and in its liver active repair was found to be taking place of a recent hemorrhagic lesion such as has already been described. When several, small, desensitizing doses or one large one were used (in guinea pigs) the results were no better. The local effect in the guinea pig has some similarity to the Arthus phenomenon, but the latter is in our experience a less severe type of lesion in this species and far more difficult to elicit, at least with horse serum, while it lacks the hemorrhages caused by the absorbed and precipitated rabbit serum.

It is well known that the development of serum sickness is accompanied in man by the appearance of precipitins in the blood, and

that the urine may show a coincident albuminuria. We have followed the urines of a number of dogs and guinea pigs subjected to severe "shock" by the intravenous injection of precipitated or unprecipitated serum which had been exhausted with red cells. None showed noteworthy urinary change. Casts were regularly absent, and the slight trace of albumin occasionally noted was no greater than was inconstantly present prior to the injection.

Despite all this, there is no denying that the effects of the serum may be due to the same toxic principle or principles concerned in anaphylaxis. But, if so, an important difference in the quantitative relations must be assumed.

Possible Sources of the Toxicity.

Is the toxic element primarily present in the serum, or is it engendered by treatment? We feel convinced that the former is the case. The "shock" produced by the absorbed, or absorbed and precipitated, serum differs in no essential from that caused by inactivated but otherwise untreated precipitating serum, as observed by Uhlenhuth and Haendel, Doerr and Moldovan, and ourselves. Were it due to hemolysins and hemagglutinins persisting after absorption, the examination of the blood and the autopsy findings would give evidence of this, while furthermore the process of repeated absorption, even if incomplete, would greatly diminish the toxicity of the serum. Neither is the case. Our serum, as already mentioned, was overabsorbed; that is, submitted to more red cells than were necessary to take out all demonstrable hemolysins and agglutinins, a process which renders completely innocuous the ordinary hemolytic and hemagglutinative serum.

Many observations were made which bore on the question of whether gross precipitation within the animal body might not be a cause of the disturbances noted. Always the precipitated serum failed *in vitro* to cause a clouding when mixed with even a little more than its bulk of guinea pig serum, far less than the preponderant quantity encountered on its injection into the blood. Such tests would seem to rule out actual precipitation as the cause of disturbance, unless indeed the conditions with plasma differ greatly from

those with serum—and there is no reason to suppose that they do, since complement is unnecessary for the precipitin reaction. We have said that the toxicity of the different sera varied in general with their original precipitin titer. Yet that the “shock” engendered by the serum had no essential dependence on the immediate precipitin content was well shown in the results with serum from which the precipitin had been removed. The possibility remains that the toxic element may be a product of the interaction of precipitin and precipitinogen, one formed as readily when the two are brought together without as within the animal body. Against this is the fact shown by Friedberger⁷ that specific precipitation *in vitro* fails to give rise to a toxic element unless complement be present; for it was absent in our experiments. But Friedberger made his tests of toxicity on animals of a species to which both precipitin and precipitinogen were strange, whereas in our work the precipitin was directed against the serum of animals of the species used for the tests, and just such serum was employed for the *in vitro* removal of precipitin.

There remains the interesting possibility of the presence in the serum of a hitherto unrecognized toxic antibody. Further work alone can justify any speculation in this direction.

SUMMARY.

There is present in serum of high precipitin titer, produced by the repeated injection of rabbits with the blood-free serum of guinea pigs or dogs, a principle highly toxic for animals of the species furnishing the antigen. Intravenously the serum causes severe shock, and even sudden death, while locally it gives rise to acute inflammatory changes and profuse capillary hemorrhages. The complete removal of hemolysins and hemagglutinins from the serum by exposing it repeatedly to washed red cells lessens its toxicity to only a slight degree and one obviously dependent on these elements; while the further removal of precipitin by specific precipitation *in vitro* has no detoxifying effect whatever. Whether the toxic principle is a hitherto unrecognized antibody or perhaps a toxic product of the interaction of precipitin and precipitinogen,—one formed as readily in the test-tube as in the animal body,—remains to be determined.

The symptoms of guinea pigs and dogs given an intravenous injection of treated or untreated serum markedly resemble those of anaphylaxis, but our attempts at desensitization have been unsuccessful. The local lesion in guinea pigs is more severe than that of the Arthus phenomenon. But these differences from anaphylaxis may, of course, be dependent merely on differing proportions of constituents that are themselves, as yet, scarcely apprehended.

Our observations, as here summed up, were made with a practical point in mind, and as regards this point they are of a discouraging nature. In papers already published it has been shown that sera specifically effective against infections of which the excitant is unknown can in some cases be obtained by using infected tissue itself as antigen. Such sera must, of course, be deprived of antibodies injurious to tissue, prior to their employment in the animal body; and this was successfully accomplished in our early experiments by exhaustion with washed red cells. The purpose of the present work was to determine whether serum used as antigen gives rise to injurious principles in the antiserum. For the serum of infected individuals would in many diseases form a convenient antigen. It is evident that injurious principles result from its use, and that they are not removed from the antiserum when the latter is exhausted with red cells and its precipitin removed by specific precipitation, nor can their action be nullified by desensitization as carried out in anaphylaxis. Unless the obstacle of their presence is in some way overcome the body fluids of infected human beings cannot be practically utilized for the production of antiserum. In test animals the difficulty is not so grave. For we have found that the toxic antiserum produces no enduring lesions when it is administered intravenously in non-lethal doses.

DETERIORATION OF CRYSTALLINE STROPHANTHIN IN AQUEOUS SOLUTION.

ITS RELATION TO HYDROGEN ION CONCENTRATION AND A METHOD FOR ITS PREVENTION.

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For clinical use crystalline strophanthin is commonly dissolved in normal salt solution or water and marketed in glass ampules. Sterilization is accomplished by autoclaving after the ampules have been filled and sealed. In making biologic assays of several lots of a commercial preparation of ouabain (g-strophanthin) by the cat method of Hatcher and Brody¹ wide variations in potency were found. On adding a drop of indicator, phenol red, to the contents of the ampules showing low potency, it was observed that they were decidedly alkaline in reaction, whereas freshly prepared, aqueous solutions of the drug were neutral or slightly acid. Experiments were undertaken to ascertain the cause of the deterioration in relation to the altered hydrogen ion concentration and to devise a method for preparing a stable solution for therapeutic purposes.

The Drug.

Great variability in commercial preparations of crystalline strophanthin has been noted by Rowe,² who tested a number of lots bought on the open market by the "1 hour" frog method. The least active sample was 67 per cent as active as the standard (U. S. P.), whereas the most active was 240 per cent of the same standard, or about 3.5

¹ Hatcher, R. A., and Brody, J. G., The biological standardization of drugs, *Am. J. Pharm.*, 1910, lxxii, 360. The cat unit represents the minimum lethal dose per kilo of cat, expressed in milligrams.

² Rowe, L. W., The variability of strophanthin, with particular reference to ouabain, *J. Am. Pharm. Assn.*, 1916, v, 1183.

times as potent. As indicated by Rowe, such variability in the crystalline product can, in large part, be attributed to the difficulty of obtaining unmixed seed of a desired variety, for there are more than twenty species of *Strophanthus* seed on the market and a given lot almost invariably contains specimens of several varieties. Further, different samples of the crystalline product contain variable amounts of water of crystallization; this, too, makes for differences in biologic activity expressed in terms of dry weight.

The drug employed in the present observations, as well as in a series of clinical studies to be reported later, was isolated from seeds purchased as *Strophanthus hispidus*, but apparently actually *Strophanthus gratus*. The procedure of isolation followed was based on the methods of Heffter and Sachs, and Thoms.³

The ground seeds were freed from fat with petroleum ether and the dried residue was extracted with cold 70 per cent alcohol. The extract was concentrated to a thick mass *in vacuo*, taken up in hot water, treated with hot basic lead acetate until no further precipitate formed, and filtered. The excess of lead was precipitated from the filtrate by means of a little ammonium sulfate and filtered off. The filtrate was saturated with ammonium sulfate, which precipitated a mixture of the amorphous and the crystalline strophanthin (ouabain), the last portions of the latter separating in crystalline form. The precipitate was dissolved in warm water, filtered, and the filtrate chilled and seeded with a few strophanthin crystals. After being allowed to stand in the ice box, the crystalline strophanthin was filtered off and recrystallized from water.

The crystals so obtained consisted of transparent, glistening platelets, containing 8.5 molecules of water of crystallization. The color reactions corresponded to those found by Arnaud and by Thoms.⁴

Physical constants:—Anhydrous substance: $[\alpha]_D^{20} = -30.2^\circ$

Calculated for $C_{30}H_{46}O_{12}$: C, 60.17; H, 7.75

Found: C, 60.05; H, 7.81

Melting point (uncorrected): Softens: above 185°C ;
melts to paste: $187\text{--}188^\circ\text{C}$.; evolves gas: $195\text{--}197^\circ\text{C}$.

³ For the isolation and identification of the drug we are indebted to Dr. W. A. Jacobs and Dr. M. Heidelberger, of The Rockefeller Institute. They have also kindly furnished us with notes of the methods employed by them in these procedures. See also Heffter, A., and Sachs, F., *Vergleichende Untersuchungen über Strophanthus-Glucoside*, *Biochem. Z.*, 1912, xl, 83; Thoms, H., *Die Strophanthus-Frage vom chemischen Standpunkt*, *Ber. pharm. Ges.*, 1904, xiv, 104.

⁴ Thoms, H., *Ber. pharm. Ges.*, 1904, xiv, 104.

Biologic standardizations of this crystalline strophanthin were carried out on thirty-one cats, and in twenty-six of the animals electrocardiograms were made at frequent intervals during the course of the experiments in order to determine the physiological effects of the glucoside upon the cat's heart. The average cat unit was found to be 0.104 mg. per kilo, a figure in accord with the standard of 0.10 mg. per kilo established by Hatcher and Brody. The electrocardiographic findings confirmed in their essentials the observations of Levine.⁵ Chemically, biologically, and physiologically, therefore, the strophanthin at our disposal possessed those properties ascribed to the crystalline product obtained from *Strophanthus gratus* and usually marketed under the name "ouabain."

Experiments with Glassware.

Doubly distilled water, pH 6.0, was autoclaved for 20 minutes at 15 pounds pressure in various types of glass bottles and flasks, chosen at random from the laboratory supply. Immediately after autoclaving, the reaction of the water in the cheaper and softer varieties of container had become distinctly more alkaline, the pH ranging from 6.3 to 9.0+.⁶ In the hard glass flasks (Pyrex) no significant alteration in reaction (less than 0.1) occurred. A standard 0.02 M phosphate mixture, pH 7.0,⁷ autoclaved in the bottles giving off the maximum amount of alkali in the process of sterilization, showed no alteration in hydrogen ion concentration, the buffer action being adequate to compensate for the dissolved alkali.

A similar experiment was done with sixteen types of glass ampules obtained from a number of pharmaceutical firms and used by them in marketing their products. The ampules were filled with distilled water, sealed, and autoclaved. In every instance the water in the

⁵ Levine, S. A., The action of strophanthin on the living cat's heart, *J. Exp. Med.*, 1919, xxix, 485.

⁶ Determinations of hydrogen ion concentration were done colorimetrically. In the more alkaline solutions the results obtained by the colorimetric method were checked by the use of the hydrogen electrode.

⁷ The standard phosphate mixtures used were prepared according to the directions of Sørensen (Sørensen, S. P. L., *Biochem. Z.*, 1909, xxi, 201; *Ergebn. Physiol.*, 1912, xii, 393).

ampules showed a change in pH, which now ranged from 6.2 to 9.0+. In order to titrate back to neutrality (pH 7.0) the most alkaline solution in the series, with phenol red as indicator, 2.6 cc. of 0.02 N hydrochloric acid per 100 cc. were required (Table I).

TABLE I.

Hydrogen Ion Concentration of Distilled Water after Autoclaving in Various Types of Glass Ampules. Initial pH=6.0.

Ampule No.	pH of contained water after autoclaving 20 min.	Amount of 0.02 N HCl required to titrate 100 cc. of water to pH 7.0.*
		cc.
1	6.7	
2	6.2	
3	7.0	
4	7.0	
5	7.0	
6	7.0	
7	7.0	
8	9.0+	1.10
9	7.0	
10	7.2	
11	7.0	
12	9.0+	2.17
13	7.0	
14	9.0+	1.35
15	9.0+	2.10
16	9.0+	2.60

* Phenol red was used as indicator.

Effect of Changes in Hydrogen Ion Concentration on Aqueous Solutions of Crystalline Strophanthin.

A 2 per cent solution of strophanthin was made in standard 0.05 M phosphate mixtures with pH 5.0, 7.0, and 8.6. Biologically tested, the cat unit of these solutions was found to be 0.107 mg. per kilo,⁸ their optical rotation -0.97° . After autoclaving for 20 minutes at

⁸ In making determinations of cat unit values at least three animals were employed to calculate the biologic activity. Each figure given, therefore, represents the average of three or more cat experiments. Where it is stated that no alteration in activity was noted, it is meant that less than a 10 per cent variation occurred, this variation representing changes in the third decimal place only.

15 pounds pressure in Pyrex flasks, no alteration in optical activity and no significant change in potency were observed in the acid or neutral solutions. The alkaline mixture, however, now had an optical rotation of -0.93° and a cat unit value of 0.152. In short, when strophanthin was autoclaved in alkaline solution (pH 8.6), the molecule was partially decomposed, with resultant alteration in its ability to rotate polarized light and significant reduction in biologic activity (Table II).

The degree of alkalinity attained by aqueous solutions autoclaved in soft glass ampules is, therefore, more than ample to cause chemical changes in their contents.

TABLE II.

Effect on Biologic Activity and Optical Rotation of Autoclaving Crystalline Strophanthin in 2 Per Cent Aqueous Solution at Different Hydrogen Ion Concentrations.

pH of 0.05 M phosphate solution.	Cat unit value per kilo.		Optical rotation.	
	Before autoclaving.	After autoclaving.	Before autoclaving.	After autoclaving.
	mg.	mg.		
5.0	0.107	0.104	-0.97°	-0.97°
7.0	0.107	0.105	-0.97°	-0.97°
8.6	0.107	0.152	-0.97°	-0.93°

For clinical use it is convenient to employ crystalline strophanthin in dilute concentration, usually 0.01 per cent. Such solutions autoclaved in soft glass ampules, which gave off enough alkali to alter the reaction of the contents from pH 6.0 to pH 9.0, became at once biologically inert, more than four times the calculated lethal dose having no appreciable effect on the cat's heart. The contents of hard glass ampules, with no significant alteration in pH after sterilization (0.1 or less), retained full potency. In hard glass (Pyrex) such autoclaved solutions have been kept without deterioration for over 5 months.

Holste⁹ noted an alkaline reaction in solutions of Boehringer's amorphous k-strophanthin which had deteriorated, and suggested that alkali given off from the glass containers might have induced hydrolytic cleavage of the glucoside.

⁹ Holste, A., Zur Strophanthinfrage, *Z. exp. Path. u. Therap.*, 1918, xix, 153.

Like Thoms,¹⁰ who observed marked reduction in toxicity for mice of sterile aqueous solutions of amorphous k-strophanthin, he found crystalline g-strophanthin to be more stable. Holste's observations, however, were carried no further, and no method for preventing deterioration was offered.

We have had made for our use tablets of g-strophanthin, 0.5 mg., triturated with sugar of milk. The amount of drug contained is so small and the percentage error in measuring out the powder for the individual tablet is, therefore, relatively so large, that this form of preparation was discarded as unsatisfactory.

A Method for Preparing Crystalline Strophanthin in Solution for Clinical Use.

It is clear that stability of reaction is essential if the potency of strophanthin in solution is to be preserved. The glass commonly used in the manufacture of ampules for commercial purposes is, in large part, of the soft variety, yielding considerable amounts of alkali to solutions contained in it. It is advisable, therefore, to put up solutions of crystalline strophanthin in hard glass containers. Ampules of Pyrex glass have proved satisfactory. In order to avoid even slight changes toward the alkaline side, the drug may best be prepared in 0.02 M standard phosphate solution at pH 7.0.¹¹ This concentration of phosphate is harmless when injected intravenously in small amounts (5 to 10 cc.) into patients and does not interfere with either the chemical or physiological properties of the glucoside. Crystalline strophanthin in such a solution, in 0.01 per cent concentration (5 cc. contain 0.5 mg.), sealed in ampules, and autoclaved, has been preserved without evidence of reduction in biologic activity for more than 5 months.

¹⁰ Thoms, H., *Alte und neue Aufgaben der pharmazeutischen Chemie und insbesondere über die biologische Prüfung der Arzneimittel*, *Ber. pharm. Ges.*, 1913, xxiii, 452.

¹¹ A 0.02 M phosphate solution at pH 7.0 contains 1.03 gm. of KH_2PO_4 and 1.76 gm. of Na_2HPO_4 (anhydrous) per liter.

SUMMARY.

1. Many of the glass containers commonly used in the laboratory, and most of the glass ampules employed in marketing sterile solutions for hypodermic or intravenous medication, yield sufficient alkali on autoclaving, to change the reaction of distilled water from pH 6.0 to pH 9.0.

2. This increase in alkalinity is sufficient to render biologically inert and partially to decompose aqueous solutions of crystalline strophanthin in the concentration ordinarily employed in the clinic.

3. It is suggested that for clinical use crystalline strophanthin be dissolved in 0.02 M standard phosphate solution at pH 7.0, and marketed in hard glass ampules, thereby insuring stability of reaction with preservation of biologic activity.

4. It should be borne in mind, both by laboratory worker and pharmacist, that the alkali yielded, on heating, by soft glass containers may be responsible for a considerable alteration in the hydrogen ion concentration of their contents.

GIANT CENTROSPHERES IN DEGENERATING MESEN- CHYME CELLS OF TISSUE CULTURES.

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PLATES 16 AND 17.

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INTRODUCTION.

In a previous communication (Lewis) on degeneration granules and vacuoles in living fibroblasts of chick embryos cultivated *in vitro*, it was noted that these granules and vacuoles, as they increase in number, tend to accumulate about the centriole which is located near one side or one end of the nucleus. Parallel with the accumulation important changes occur in this region of the cell. A clear area, gradually increasing in size, may develop about the centriole. This area, the centrosphere, may become as large or larger than the nucleus and is usually almost entirely free from degeneration granules and vacuoles. The latter form a corona about it, varying in width in different cells according to the number of granules and vacuoles which have accumulated.

Attention was also called to the fact that the mitochondria usually become arranged, more or less radially, about the centriole and the centrosphere, and that they may lie partly in the cytoplasm between the granules and vacuoles and partly in the clear peripheral region which is free from granules and vacuoles. In cells in which no distinct radial arrangement of the mitochondria about the centrosphere was seen and in which the mitochondria were usually in the form of short rods and granules, it was almost always noticed that the mitochondria in the cytoplasmic strands between the vacuoles were more abundant about the centrosphere than about the nucleus.

It is about the centriole or centrosphere, not about the nucleus, that the granules and vacuoles and the mitochondria are orientated.

In the above type of degeneration vacuolation of the cytoplasm is the predominant feature, but in the type considered in the present paper the enlargement of the centrosphere is the most characteristic change. The two forms of degeneration are not sharply separated from one another, and all gradations between them are found in the mesenchyme cells. We are uncertain whether these variations in the behavior of the cells during degeneration are dependent upon a difference in the type of mesenchyme cell or upon differences in the individual cells themselves, combined with differences in the cultural environment. In cultures of the same region sometimes one type predominates and sometimes the other. The two types may be characterized as follows:

Vacuolar Type (Fig. 1).—(1) Extensive vacuolation about the centrosphere and finally of the entire cytoplasm. (2) Enlarged centrosphere, usually without a sharp border or medullary zone. (3) Radial arrangement of mitochondria about the centrosphere.

Giant Centrosphere Type (Figs. 3 to 8).—(1) Slight or moderate vacuolation about the centrosphere. (2) Enlarged centrosphere with sharp border, centriole, and medullary and cortical zones. (3) Concentric or radial arrangement of mitochondria about the centrosphere.

The term centrosphere is used to designate the peculiar differentiated region about the centriole. It corresponds to the centrosphere or central body, the aster or astrosphere, of the dividing egg and cell. The terminology of this whole central apparatus is in a chaotic state and I have tried to select names that will not confuse. The centriole, when present, seems to be always in the form of a granule; it may appear to be single or double, usually the latter. Often the centrosphere consists of one or more zones which surround the centriole. These zones have been designated in various ways by different authors, but their significance and homologies are very obscure. The centrosphere in its most complete form, as seen in the fixed material, consists of a centriole, single or double, immediately surrounded by a clear medullary zone, and beyond this a large cortical zone. A granular zone, varying in thickness, may sometimes be seen between the medullary and cortical zones. This, however, is probably more or less transitory and accidental and is not to be considered as strictly

a part of the central apparatus. The cortical zone is surrounded by an outer granular zone, but this, also, is probably not strictly a part of the centrosphere. Both of the granular zones, inner and outer, consist, for the most part, of degeneration granules. The cortical zone may be sharply limited or may continue out into the cytoplasmic framework between the vacuoles. Its exact boundaries in this condition are difficult to define.

Distinct astral rays are not seen either in the living or in the fixed specimens, and they are also absent in the more normal dividing cells. Since in the fixed material the spindle fibers stand out clearly, it is probable that the astral ray fibril substance is either not present in these cells or is not laid down in such a manner as to appear, on coagulation, as radiating fibers. Often a more or less coarse radiation of the unvacuolated cytoplasm extends out from the centrosphere towards the periphery between the vacuoles, but it does not correspond to the astral rays.

The movements of the vacuoles and granules between centrosphere and periphery, or *vice versa*, have been described elsewhere in considerable detail (Lewis). The moving granules and vacuoles do not penetrate into the centrosphere but stop and become collected more or less at the periphery. This peculiar behavior of the granules would seem to indicate that the centrosphere is a semisolid gel or is surrounded by a membrane into which the granules cannot penetrate, while outside this is a semifluid in which granules and vacuoles move or are moved about by currents of cytoplasm.

Chambers concludes from his microdissection studies that "the development of the amphiaster is associated with the formation of two semisolid masses within the more fluid egg substance." Since these semisolid masses are probably centrospheres and correspond to those of the degenerating cells in the present study, Chambers' work lends support to the idea that the enlarged centrospheres of degenerating cells are also semisolid.

In the vacuolated type of cells it seems probable that the irregular cytoplasmic framework extending out from the centrosphere is also of a semisolid consistency and connects the semisolid centrosphere with a more or less semisolid peripheral layer, and that the vacuoles and granules occupy the more fluid part of the cytoplasm in which

are produced currents that carry the passive granules back and forth. Such currents might result from changes in osmotic conditions, due to various metabolic states. The mitochondria lie in the cytoplasmic framework between the vacuoles and are usually more or less radially arranged about the centrosphere. Their movements are different in character from those of the granules, as one would expect them to be on the theory that they lie in a more solid framework.

In the type of cells with enlarged centrospheres, an intervacuolar framework is not so apparent, but there is usually concentrated about the centrosphere a more deeply staining cytoplasm in which lie mitochondria and granules and vacuoles, while the more peripheral part of the cytoplasm stains very lightly or not at all.

Normal Mesenchyme Cells in Tissue Cultures.

In fixed and stained specimens of the first 20 hours most of the cells appear normal or nearly so. The centriole can often be recognized without difficulty. It is usually double and lies near the nucleus, at the side or at one end, more often at the side. It may be near the center of one side or towards either end. Often it lies within an indentation of the nucleus which occasionally is so deep that the double centriole appears to be within the nucleus. There is little or no radial arrangement of the mitochondria except such as usually occurs in multipolar cells in which the long mitochondria extend out into the processes. There is usually, however, more cytoplasm on the side of the nucleus which includes the centriole, and it also appears to be denser. There are always cells in a culture in which one cannot determine the centriole with absolute certainty, and others in which no structure corresponding to it can be made out. A few cells in cultures incubated for a period of 24 hours show the beginnings of a radial arrangement of the mitochondria and an increase in the number of fine granules about the centriole. The mitochondria are long, thread-like, and often branched. They never seem to exhibit any definite relation to the nucleus, and only occasionally, as mentioned above, to the centriole.

Degenerating Mesenchyme Cells.

The three cultures described below have been selected from among others in which the enlarged or giant centrosphere type of degeneration predominates. The first culture, No. 404, shows many transitions between more or less normal cells and those with enlarged centrospheres, while the second culture, No. 441, shows, for the most part, only cells with enlarged centrospheres. The degenerative processes have gone still further in No. 444, in which many of the centrospheres have lost their centrioles and medullary zones, and granules and vacuoles can be seen in the centrosphere itself.

Culture 404 (Text-Figs. 1 to 9).

This culture is from an explant of subcutaneous tissue and muscle of the leg of an 8 day chick embryo cultivated in Locke-Lewis solution (Locke's solution plus 0.25 per cent dextrose plus 10 to 20 per cent chicken bouillon). The growth was abundant. After 48 hours the culture was fixed in Zenker's fluid, without acetic acid. The preparation was stained lightly with iron-hematoxylin.

Most of the cells show signs of degeneration but there is little vacuolation of the cytoplasm, even where the centrosphere has become as large as the nucleus. Various stages in the enlargement and differentiation of the centrosphere are to be found associated with changes in the arrangement of the mitochondria and the accumulation of fine granules, probably degeneration granules, about the centriole and centrosphere. Degeneration seems, as a rule, to be more advanced near the periphery of the growth than near the explant.

In a sector of the outer two-thirds of the growth about one-third of the cells show large centrospheres, one-third small and medium sized ones, and in about one-third of the cells no centrospheres are found. The mesenchyme cells are mostly multipolar in character, and such as are usually found in cultures associated with the outgrowth of skeletal muscle.

In the earlier stages of degeneration there is no accumulation of special centrosphere material about the centriole. The fine degeneration granules gather more or less about the centriole and the mitochondria tend to become more or less radially arranged about it. As

Text-figs. 1 to 9, from Culture 404, show stages in the development of the centrosphere.

TEXT-FIG. 1. Accumulation of degeneration granules about the centriole.

TEXT-FIG. 2. Appearance of a clear medullary zone about the centriole with a corona of degeneration granules and radial mitochondria.

TEXT-FIG. 3. Beginning cortical zone formation; mitochondria separated from the granular zone.

TEXT-FIG. 4. The same condition except that the mitochondria are short and thick and concentrated more about the cortical zone.

TEXT-FIG. 5. Well developed centrosphere with the centriole, medullary and cortical zones, inner granular zone, and degeneration granules about the cortical zone. The mitochondria are becoming concentric.

TEXT-FIG. 6. Inner granular zone gone. The medullary zone stains more deeply than the cortical zone.

TEXT-FIG. 7. The mitochondria are mostly concentric about the centrosphere; there is none about the nucleus.

TEXT-FIG. 8. The medullary zone is disintegrating and the centriole is not visible.

TEXT-FIG. 9. The medullary zone and centriole have disappeared.



the process goes on there gradually develops about the centriole an area free from degeneration granules, vacuoles, and mitochondria (Text-figs. 1 to 4). This clear area never becomes very large and is surrounded by a halo of very fine granules. It forms, I think, the medullary zone of the large complete centrosphere. The mitochondria, which are arranged more or less radially about this area, at first have their inner ends among the granules which surround it. There are many points about the clear medullary zone that are still obscure—whether it is fluid or solid, and what its origin is; or whether it is a centriole vacuole, pushing away the degeneration granules from the centriole as it enlarges. If it is a fluid it must have some sort of a limiting membrane. There is no special reason to suppose that it comes from the granules which at an earlier stage closely surround the centriole. It develops very much like a vacuole about a granule but in the living cell it never takes up the neutral red or other vacuolar dyes, such as methylene blue (Ehrlich) or brilliant cresyl blue. The granules which surround the periphery of the medullary zone obscure its outline more or less; later, when these granules disappear, as in Text-figs. 6 and 7, a sharp border or thin membrane separates it from the cortical zone. This membrane is not always homogeneous. The medullary zone is often eccentrically placed. In the early stages the medullary zone does not seem to take up the hematoxylin stain, but in later stages, after the granular zone has disappeared, the medullary zone is often stained darker than the cortical zone. In what I judge to be still older stages, the medullary zone becomes very obscure or may disappear altogether. Sometimes faint irregular traces of it are seen. The centriole also disappears in these more advanced stages or, at least, fails to stain, for no trace of it can be found (Text-figs. 8 and 9).

The details of the formation of the cortical zone are just as obscure as those of the medullary zone. There is a gradual accumulation of cortical substance about the medullary zone and the fine granular zone which immediately surrounds it. The mitochondria seem to be pushed away from the medullary zone (Text-figs. 3 and 4). Ultimately there is formed a large cortical zone which may give to the centrosphere a size about that of the nucleus (Text-figs. 5 to 9). The origin and nature of this cortical substance are uncertain. It

seems probable that it consists of the more or less hypothetical substance known as archoplasm, kinoplasm, spongioplasm, etc., and there is evidence that it has been drawn into the centrosphere from the surrounding cytoplasm.

In the early stages we have seen that the long mitochondria radiate from the centriole and centrosphere far out into the cytoplasm. Some significant changes take place, more or less parallel with the increase in size of the centrosphere. The mitochondria tend to accumulate more and more immediately about the enlarging centrosphere and the radial arrangement gradually gives place to a more or less concentric arrangement, as seen in Text-figs. 4 to 9. The mitochondria are probably not actively motile bodies but dependent upon movements of the cytoplasm. I picture the stages of the development which takes place somewhat in this order. There is a drawing or a flowing in of a certain part of the cytoplasm, presumably the archoplasm, to the centrosphere. This flowing in of the archoplasm in the earlier stages helps to explain the usual more or less radial arrangement of the mitochondria. They are pulled in, somewhat reluctantly as it were, towards the center, and thus tend to become extended out in radial lines. In the early stages of the process the cortical zone is without sharp boundary and extends out into the cytoplasm. There is evidently some sort of transformation of the archoplasm as it accumulates about the medullary zone. This may be merely a change to a more semisolid consistency or to a condition such as would stop the mitochondria at the periphery and also prevent the penetration of new granules and vacuoles which accumulate around it. Finally, most of the archoplasmic material becomes concentrated within the centrosphere or immediately about it, and the outlying cytoplasm appears to be clear and devoid of granules and mitochondria. The substance that is finally concentrated within the centrosphere is different from that immediately about it and from that in the more peripheral regions of the cell. The substance immediately about the centrosphere is also peculiar. It contains the mitochondria and granules and is itself finely granular and stains more deeply than the peripheral cytoplasm. These differences can be seen in the living, as well as in the fixed and stained material.

The granular zone is variable and probably consists merely of degeneration granules which in the living cell take the neutral red.

What might be called an inner granular zone lies between medullary and cortical zones; this is not constant and is probably not a part of the centrosphere proper.

In early stages, as we have noted, degeneration granules, which take up neutral red in the living cell, accumulate about the centriole. It seems probable that the developing medullary zone pushes them back from the centriole. The gradually enlarging medullary zone is always surrounded by a halo, composed at first of fine, later of coarse granules. As the cortical zone develops this halo diminishes. Some of the granules appear to be pushed to the periphery of the gradually enlarging cortical zone where other newly formed granules have been accumulating. A few scattered granules are often seen in the cortical zone. All conditions between a well marked inner granular zone and its complete absence can be found. In many cells the granules disappear ultimately, either dissolving or finding their way to the periphery of the cortical zone.

The mitochondria, which are at first long and thread-like and often branched, gradually break up into short threads, rods, and granules which, in turn, may swell up into vesicle and other polymorphic forms. In general the changes parallel those in the centrosphere, but not always, indicating that the changes are more or less independent of each other. It is obvious that the mitochondria accumulate about the enlarging centrosphere and also that the central ends of many are pushed back from their original position at the periphery of the medullary zone to the enlarging periphery of the cortical zone. The latter process would also tend to change their position from radial to concentric. Fig. 2 illustrates a stage in the concentration of the archoplasm about the centriole. This large, flat, mesothelial-like cell consists of a broad, scarcely stainable, peripheral layer of cytoplasm and a more central, finely granular area that appears to be closing in about the centrosphere, which consists of a centriole and small medullary zone.

Culture 441 (Figs. 3 and 4).

This culture is from an explant of a piece of a large blood vessel of a 6 day chick embryo in Locke-Lewis solution. The growth was abundant. It was fixed after 3 days in Zenker's fluid, without acetic

acid, and deeply stained in iron-hematoxylin. Most of the cells show large centrospheres that are smaller than the nucleus. Of 44 cells, carefully examined, 7 showed a centriole, medullary zone, and cortical zone; 10, medullary and cortical zones, the centriole being doubtful; 13, only medullary and cortical zones with no trace of a centriole; 14, only cortical zone material. A few of the cells showed vacuolation of the centrosphere. The centrospheres in these cells are more like those usually pictured as cancer cell inclusions. They have a light cortical zone and a deeply staining medullary zone. There are practically no transition stages, as shown in Culture 404.

The centrospheres are rather sharply outlined except where the surrounding denser part of the cytoplasm with its granules, vacuoles, and mitochondria obscures the edge. The material of the centrosphere is also different in character from the rest of the cytoplasm. The latter shows a moderate degree of vacuolation about the centrosphere. The mitochondria vary from rods and threads to granules and are more or less radially arranged about the centrosphere.

Culture 444 (Figs. 5 to 8).

This culture is from an explant of subcutaneous tissue and muscle of an 8 day chick embryo in Locke-Lewis solution. The growth was abundant. It was fixed after 48 hours in Zenker's fluid, without acetic acid, plus a few drops of 2 per cent osmic acid. The preparation was deeply stained with iron-hematoxylin and counterstained with Bordeaux R.

Most of the cells in the culture show a small amount of vacuolation and a large centrosphere which has reacted in a characteristic manner to the fixation and staining, so that it has a different texture from the rest of the cell cytoplasm. Many of these centrospheres have a clear-cut margin which defines them from the surrounding vacuolated cytoplasm, but there is apparently no visible membrane about the centrosphere. In a count of 100 cells, in 8 the centrosphere was larger than the nucleus, in 18 about the same size, in 70 smaller than the nucleus, and in 4 cells a centrosphere was not recognizable. The medullary zone and centriole are either almost invisible or have disappeared entirely. Vacuoles in the centrosphere are frequent, and tend to obscure and confuse the picture. In only 4 of the 100 cells

carefully examined did the centrosphere seem to be entirely free from vacuoles. These are usually at or near the center of the centrosphere but not symmetrically placed as a rule. A vacuole within the centrosphere, with a granule lying near its center, is often difficult or impossible to differentiate from the medullary zone and its centriole. Most of the centrospheres also contain granules. These are usually smaller than, but sometimes as large as mitochondrial granules, and in fixed material, stained in the manner described, it is impossible to differentiate between them. In living material, stained with neutral red and Janus black No. 2, mitochondrial granules are rarely seen within the centrospheres. Granules which take up the neutral red, however, do occur, and it is probable that the granules in these centrospheres are of this type and are to be considered as degeneration products. There is the possibility that some of the granules may represent centrioles and that a multiplication of them has occurred, such as was found by Heidenhain in certain giant cells. The granules are usually most numerous near the center of the centrosphere and also in that part of it next to the nucleus, as seen in Fig. 8. Sometimes they are arranged in a ring around a central clear area, possibly the medullary zone (Fig. 7). The difficulty or impossibility of distinguishing the various types of granules in specimens stained with iron-hematoxylin should certainly guard one against too free speculation as to their true nature. It is only because I have examined hundreds of living cells doubly stained with neutral red and Janus black No. 2 or Janus green that I venture even to suggest the nature of the granules.

The vacuolation of the cytoplasm is, as a rule, of moderate degree only and is confined, for the most part, to the region immediately about the centrosphere. The thin clear peripheral part of the cytoplasm is usually entirely free from vacuoles as in Fig. 8. It is about the centrosphere rather than about the nucleus that the vacuoles are found, as most of the figures show. It is not uncommon for the vacuoles to extend entirely around the centrosphere, even between it and the nucleus. The nucleus is usually at one side or near one end of the cell while the greater mass of the cytoplasm, with its vacuoles and granules, surrounds the centrosphere. This is occasionally much emphasized as shown in Fig. 6 in which the nucleus is widely separated

from the centrosphere and its surrounding cytoplasm by an elongated narrow stalk. The nucleus has very little cytoplasm about it and no vacuoles or mitochondria.

In these preparations the degeneration granules are sometimes difficult or impossible to distinguish from the mitochondrial granules. Many of the granules, which are found among the vacuoles and around the centrosphere are, however, probably mitochondrial granules or small mitochondrial vesicles.

The mitochondria are usually concentrated about the centrospheres. The majority of the cells contain only the small granular type. Other cells show both the rod and granular forms (Fig. 5), and a few show threads, rods, and granules (Fig. 8). The transformation of the normal thread-like mitochondria into rods and granules usually more or less parallels the progressive degenerative changes (vacuolation of the cytoplasm and enlargement of the centrosphere), but this is not always the case, and the two processes are more or less independent of each other.

No two cells degenerate in exactly the same manner. Each cell in the culture possesses its own peculiar individual features by which it can be distinguished from any other cell, and the degenerative changes tend to emphasize these differences. In studying a culture of this character we find ourselves dealing with individuals whose peculiarities depend only partially upon environment, especially in cases where the cells are side by side. The environment may play an important part, however, in different regions of the same culture, for the conditions in the neighborhood of a cell located at the periphery of the growth vary markedly from those surrounding a cell near the explant. In cells which have approximately the same environment as, for instance, where they lie side by side, the individual differences between them must be due, for the most part, to differences of the cells themselves, such as the interrelations in size and space and the interactions between nucleus, cytoplasm, mitochondria, centriole, etc.

Relation to Cancer Cell Inclusions.

The enlarged centrospheres in these degenerating cells are of peculiar interest because of their similarity to certain of the cytoplasmic inclusions of cancer cells (Plimmer's bodies, bird's eye inclusions, cancer parasites, cancer cell inclusions, etc.).

20 years ago and more these peculiar bodies found in cancer cells attracted much attention because of the claim by Plimmer and others that they were the cancer parasites. Plimmer found these bodies in 1,130 out of 1,278 cases. Previously, Pianese had stated his belief that the cancer cell inclusions were due to secretions. Later Borrel advanced the theory that these peculiar cytoplasmic inclusions were similar to the centrosomes of normal cells. His attention was attracted by the similarity of the centrosome apparatus of the spermatocytes of the guinea pig to the structures in the cancer cells; and he considered the cancer cell inclusions as typical centrospheres. LeCount states:¹ "The points of similarity between these bodies and the archoplasmic structures of normal cells, a likeness that had been indicated by Borrel, lead me to stop further search in the regional metastatic growths for this 'cancer parasite.' The analogies are not simply those of morphology and position, but also of affinity for dyes." LeCount's description of the cancer bodies can be applied almost verbatim to the giant centrospheres in our cultures. There are certain minor differences, as can be seen by comparing figures, but the essentials are similar. This is likewise true with the figures of Borrel, of Pianese, and of Greenough. Concerning Plimmer's bodies, the last mentioned author concludes that "their appearance, staining reactions, and situation in the cell, are such as to justify the hypothesis that they are the result of the secretory activity of the epithelial cell."

Many of the "*Vaccinekörperchen*," as figured by Hückel, seem to be typical centrospheres, often with medullary and cortical zones and with or without a centriole.

It is well known that cancer cells are subject to degenerative changes such as occur in other tissues, and if it is true, as I believe, that the enlarged centrosphere in the mesenchyme cells of the present cultures is one of the expressions of cell degeneration, it is not surprising that similar bodies occur in both types of cells. The fact that an enlargement of the centrosphere occurs in the degeneration of embryonic cells suggests that the appearance of similar centrospheres in cancer is an indication that cancer cells are likewise embryonic in character and might be used in support of the theory that cancers arise from embryonal rests.

In cancer we are dealing with epithelial cells and it is somewhat surprising, at first glance, to find such an important phenomenon as the enlargement of the centrosphere common to such different types of cells as epithelium and embryonic mesenchyme. It is probable that the fundamental metabolic processes and the mechanism for them

¹ LeCount, p. 386.

are essentially similar. If my interpretation of Hückel's figures is correct, the enlargement of the centrosphere in degeneration is not confined to cancer or to embryonic cells and may be looked for in other pathological conditions of the body tissues. The fact that it occurs in the cells of tissue cultures where, to a certain extent at least, we can control the environment, brings within the realm of experimental solution the factors which cause this condition. The cultures in which it has been found were made in the usual manner and the factors involved in producing this type of degeneration, with an especially large and well defined centrosphere, are not known. All the cells in all the cultures, unless they are washed repeatedly in fresh media, degenerate and die in from 2 to 15 days, and it is not uncommon to find giant centrospheres in degenerating cultures regardless of the age of the culture.

The Centrosphere.

We are safe in assuming that these cells with vacuoles, granules, and enlarged centrospheres are not normal, and since they die sooner than the more normal cells, these changes may be looked upon as degeneration changes. The enlargement of the centrosphere is probably an active response to the peculiar environment of the cell. The factors concerned have not been analyzed but it will probably be possible to do so experimentally. The analogy between this particular enlargement of the centrosphere and that characteristic of cell division is suggestive, in that the causal factors are probably identical or nearly so. In the normal tissue cell division results and the metabolic balance is restored; in the abnormal cells other factors prevent this and death results. Embryonic cells are repeatedly passing through a cycle of growth and division determined by the metabolic processes which take place within the cell. The enlargement of the centrosphere is one phase of this.

The material composing the centrosphere gathers about the centriole as though the latter were the dynamic center of the cell, as suggested by Boveri. The origin of this material is uncertain. In the larger centrospheres it looks and behaves differently from the rest of the cytoplasm. Whether it is chemically or physically altered cytoplasm or a concentration of the so called archoplasm or altered archoplasmic material remains to be determined.

SUMMARY.

1. Degenerating mesenchyme cells in tissue cultures cultivated in various modifications of Locke's solution often have giant centrospheres.

2. These giant centrospheres develop gradually around the centriole and in their most complete condition consist of centriole, medullary zone, and cortical zone.

3. Preceding the appearance of the centrosphere the centriole is surrounded by degeneration granules or granules and vacuoles, and as the centrosphere develops these are forced first to the periphery of the medullary zone and later, when it develops, to the periphery of the cortical zone.

4. The mitochondria become orientated about the centriole and centrosphere, at first more or less radially, later concentrically. They change from threads to rods and granules.

5. The giant centrospheres and cancer cell inclusions (Plimmer's bodies, etc.) are identical.

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EXPLANATION OF PLATES.

PLATE 16.

Figs. 3 and 4 have been retouched.

FIG. 1. Vacuolar type of degeneration in mesenchyme cells. 3 day culture from the leg of a 7 day chick embryo. Culture 406. Zenker's fluid, without acetic acid, was used for fixation, iron-hematoxylin for staining. $\times 1,250$.

FIG. 2. Concentration of the archoplasm (?) about a small centrosphere with a centriole in mesothelial-like cells of a 5 day culture from the leg of an 11 day chick embryo. Culture 440. Zenker's fluid, without acetic acid, was used for fixation, iron-hematoxylin for staining. $\times 550$.

FIG. 3. Mesenchyme cell with a large centrosphere showing cortical and medullary zones; no centriole. Mitochondria—more or less radial threads, rods, and granules—are shown. A few vacuoles may be seen about the centrosphere. 3 day culture of mesenchyme from a large blood vessel of a 6 day chick embryo. Culture 441. Zenker's fluid, without acetic acid, was used for fixation, iron-hematoxylin for staining. $\times 1,250$.

FIG. 4. Mesenchyme cells from the same culture (No. 441). The cell on the left has a large centrosphere showing cortical and medullary zones; no centriole; mitochondria, rods and granules about the centrosphere; vacuoles few. The cell on the right has a large irregular centrosphere of cortical substance only and somewhat vacuolated. It is surrounded by mitochondria and vacuoles. $\times 1,250$.

PLATE 17.

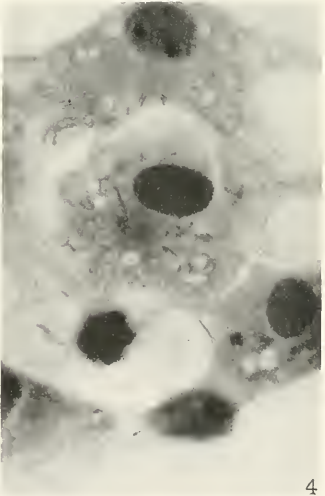
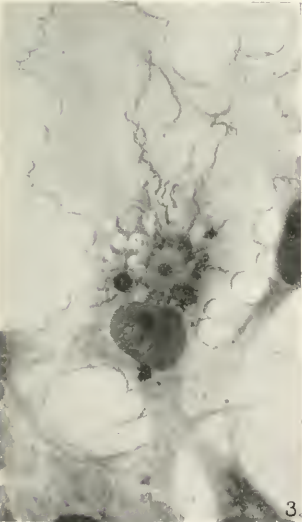
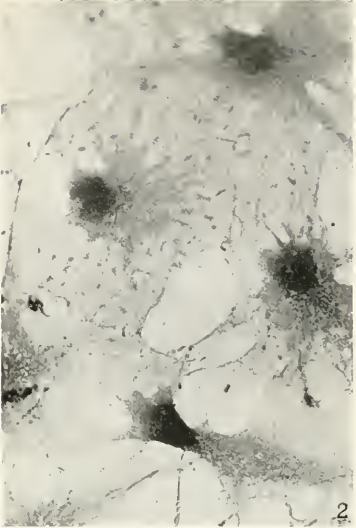
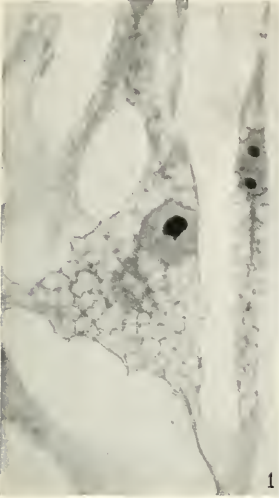
Figs. 5 to 8 have been retouched.

FIG. 5. Mesenchyme cells from Culture 444, 2 day culture from the leg of an 8 day chick embryo. The lower cell shows a large oval centrosphere with sharp outline containing vacuoles and granules; mitochondria—short threads and rods—more or less concentric; very few vacuoles. The upper cell shows a similar but smaller centrosphere surrounded by mitochondrial granules and few vacuoles. These centrospheres seem to consist of cortical substance only. Zenker's fluid, without acetic acid, plus a few drops of 2 per cent osmic acid was used for fixation, iron-hematoxylin and Bordeaux R for staining. $\times 1,250$.

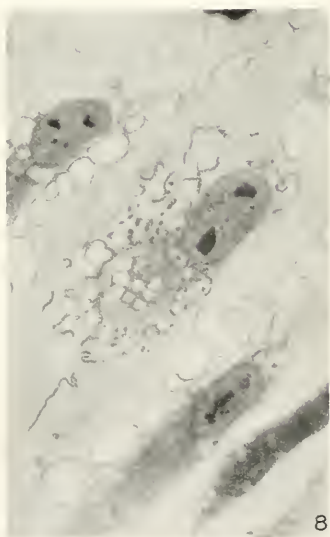
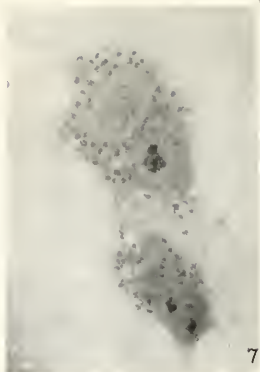
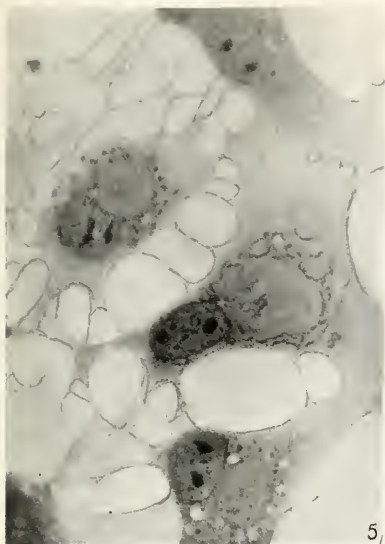
FIG. 6. Mesenchyme cells from Culture 444. The lower cell shows a large, well defined centrosphere. It appears to have a medullary zone surrounded by granules and a large cortical zone. Mitochondrial rods and granules surround the centrosphere. In the upper cell the nucleus is separated by a long, slender stalk from the body of the cell, in which is seen the centrosphere surrounded by mitochondrial granules and a few vacuoles. The centrosphere seems to consist of cortical substance only. $\times 1,250$.

FIG. 7. Mesenchyme cells from Culture 444. The upper cell shows a large centrosphere. The medullary zone with a centriole surrounded by granules is seen. The cortical zone contains scattered granules and is surrounded by granular mitochondria and vacuoles. The lower cell is smaller and contains a smaller nucleus and smaller centrosphere. $\times 1,250$.

FIG. 8. Mesenchyme cell from Culture 444. The large centrosphere shows many vacuoles and granules; no medullary zone or centriole. The mitochondrial threads, rods, and granules are more scattered than usual. $\times 1,250$.



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(Lewis: Tissue cultures.)

THE FORMATION OF VACUOLES DUE TO BACILLUS TYPHOSUS IN THE CELLS OF TISSUE CULTURES OF THE INTESTINE OF THE CHICK EMBRYO.

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PLATES 18 AND 19.

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Lewis (1919, *b*) has shown that the formation of vacuoles in the cells of tissue culture is a phenomenon frequently associated with degeneration. It was not surprising, therefore, to find that the presence of bacteria in the culture resulted in a similar behavior of the cells. While several species of bacteria did not cause the vacuolation of the cells, others did bring about this reaction. None of these, however, caused the process to take place so rapidly or so extensively as did the typhoid bacillus.

Technique.

The cultures of bacteria were grown on agar slants.¹ The inoculation was made by touching a platinum wire against the growth of organisms and then inserting it into the hanging drop of the tissue culture. The results of the experiments were extremely complicated and only one phase, the formation of vacuoles, will be discussed in the present paper.

Pieces of the intestine of chick embryos (7 to 9 days incubation) were explanted into Locke-Lewis solution (sodium chloride 0.9 per cent plus potassium chloride 0.042 per cent plus calcium chloride 0.025

¹ Transplants from the stock cultures of the Department of Pathology of the Johns Hopkins University were obtained through the kindness of Dr. L. D. Felton. All cultures of bacteria were prepared by Mr. D. T. Smith, who kindly tested out every culture of typhoid used in these experiments.

per cent plus sodium bicarbonate 0.02 per cent plus dextrose 0.25 per cent plus chicken bouillon 15 per cent) by means of the usual tissue culture technique. The growth of this particular series of cultures was among the best so far obtained in the laboratory.² Practically every culture grew luxuriantly. Not only was there abundant migration of connective tissue cells, but large membranes extended out from the endodermal lining of the intestine and also from the mesothelium. Frequently an extensive plexus of sympathetic nerve fibers was present in the new growth. These cultures, unlike those described by Lewis (1919, *b*), lived from 4 to 6 days without signs of degeneration, and only in the oldest cultures (8 to 10 days) did the number of vacuoles become marked.

The cultures of the intestine were carefully examined after 24 or 48 hours, not only in regard to the size and extent of the growth, but also as to the condition of the individual cells, especially concerning the presence of vacuoles. Any culture containing cells in which vacuoles had already appeared was discarded. This happened in only a few instances. After the preparation had been thus examined the cover-slip was removed and the hanging drop was inoculated with *Bacillus typhosus*. The number of organisms carried over was necessarily large in proportion to the amount of tissue. The culture was then resealed, turned upside down for a few seconds in order to distribute the organism, and returned to the warm box (39°C.). The controls were prepared in the same manner except that the platinum point, instead of carrying bacteria, was sterilized by passing it through the flame.

RESULTS.

As shown by Table I, it is evident that the introduction of *Bacillus typhosus* into the hanging drop of the tissue cultures resulted in rapid vacuolation of the cells of the growth (Figs. 1 to 4). These vacuoles appeared as minute round bodies which rapidly grew larger and in certain instances fused together to form large vacuoles (Fig. 4). In the ordinary degeneration of the cultures, such as described by Lewis

² Dr. Mary J. Hogue kindly made for me a series of cultures of the intestine of the chick embryo.

(1919, *b*), not all the cells became vacuolated at the same time or to an equal extent, but in these infected cultures all the cells of the cultures suffered this change and all were in the same stage of vacuolation at the same time (Figs. 1 and 2). This phenomenon was delayed when the culture was bathed with fresh solution before inoculation. It was also delayed if, instead of touching the hanging drop with bacteria, one suspended the organisms in fresh medium and placed a drop of this upon the cultures. Vacuolation, however, took place within a short time after inoculation (10 to 12 hours), even in the fresh solution, regardless of the age of the culture, so that it seemed as though the typhoid bacilli acted in such a way as to hasten markedly some process which resulted in the vacuolation of the cell.

The vacuoles were usually collected at one side of the nucleus around a central mass of cytoplasm (Fig. 3) so as to suggest that this central part corresponds to the centrosphere described by Lewis (1919, *a*). The vacuoles frequently appeared to be free from granules or to contain only minute ones. In other instances one or more granules were present.

About an hour after inoculation one or two organisms were sometimes observed within a single vacuole in one of the cells of the growth. After a longer interval, not only did more vacuoles contain bacilli but the number of these to a single vacuole was greater (Fig. 3). The vacuoles were confined to the cytoplasm of the cell and neither vacuoles nor bacteria were observed within the nucleus. While the proportion of cells containing organisms was always small, such cells were not limited to any one type of tissue, appearing in the connective tissue as well as in the mesothelium and even in the endoderm from the lining of the intestine (Figs. 1 to 3). Jones and Rous carried out observations in regard to the ability of isolated connective tissue cells from either rat or chicken cultures to ingest *Staphylococcus pyogenes albus*. They claim that such isolated connective tissue cells rarely take up carmine granules and that the phagocytosis of the bacteria occurred only in the presence of serum (antistaphylococcus). No mention is made of vacuoles in these cells.

In almost all cases in which the inoculated cultures were kept for several hours the typhoid bacilli remained motile and increased in number. Smyth performed an extensive series of experiments in

TABLE I.

Culture.	Bacteria.	Observations.				Later observations.
		After 10 min.	After 30 min.	After 1 hr.	After 2 hrs.	
1. I ₈ 48 hrs.*	12 hr. culture of No. 80 B.	Several very small vacuoles appear in all cells of growth. Some mitotic figures.	Many vacuoles present in all cells. No mitotic figures.	Many vacuoles in all cells; larger and fewer in connective tissue cells than in other cells.	In 5 of the cells one vacuole in each contains bacteria. Vacuoles large and few in number in connective tissue. Many small vacuoles in each cell of endodermal membrane and mesothelium. No mitosis.	Fixed and stained at end of 2½ hrs. (Figs. 3 and 4).
2. M ₈ 72 hrs.	3 day culture of No. 80 B.		Many vacuoles.			
3. M ₈ 96 hrs.	" "	Many vacuoles. Fixed and stained.				
4. M ₈ 48 hrs.	" "	A few vacuoles in each cell. A number of mitotic figures.	A number of vacuoles.		No more vacuoles than were present at end of half hour.	
5. I ₈ 96 hrs.	" "	A number of small vacuoles in each cell.				

6. I ₈ 7 days.	3 day culture of No. 80 B.	A few vacuoles in each cell.	A number of vacuoles.	Full of vacuoles.	Number of vacuoles not greatly increased.	Culture dead after 20 hrs.
7. I ₈ 48 hrs.	6 day culture of No. 80 B.					
8. " "	" "		A few vacuoles in each cell.			At end of 4 hrs. a few small vacuoles in each cell.
9. " "	No bacteria.		No vacuoles in the cells.			At end of 4 days growth large and only occasional cell had vacuoles.
10. I ₈ 48 hrs.	1 drop of 10 day culture of No. 80 B in Locke-Lewis solution.		A very few small vacuoles in each cell.			At end of 20 hrs. all cells dead. Cytoplasm is composed largely of a network with here and there the walls of the vacuoles still present.
11. " "	" "		" "			" "

* I₈ 48 hrs. indicates a culture of the intestine of an 8 day chick embryo, 48 hours after explantation; M₈ 72 hrs., a culture of the muscle of an 8 day chick embryo, 72 hours after explantation, etc. 80 B, etc., is the departmental number of the typhoid culture. Cultures 20 to 24 were bathed with a fresh solution before inoculation. Nos. 37 to 40 and 59 to 66 were washed with Locke-Lewis solution in which neutral red (1:50,000) had been dissolved, and later inoculated. In Nos. 10 to 18 the organism was suspended in Locke-Lewis solution and a drop of this placed on the culture. Nos. 35 and 41 to 45 were stained with neutral red in Locke-Lewis solution some time after inoculation.

TABLE I—Continued.

Culture.	Bacteria.	Observations.			
		After 10 min.	After 30 min.	After 1 hr.	After 2 hrs.
12. 1, 48 hrs.	1 drop of 10 day culture of No. 80 B in Locke-Lewis solution.			A few vacuoles in the cells.	
13. "	" "			" "	At end of 20 hrs. all cells dead. Cytoplasm is composed largely of a network with here and there the walls of the vacuoles still present.
14. "	" "		A very few small vacuoles in each cell.		" "
15. "	" "		" "		" "
16. "	" "		" "		" "
17. "	" "		" "		" "
18. "	" "			A few vacuoles in the cells.	" "

19	1, 48 hrs.	Drop of Locke-Lewis solution.				No vacuoles in cells.	At end of 4 days good growth. No vacuoles.
20.	1, 72 hrs.	18 hr. culture of No. 80 B M.				No increase in vacuoles. Bacteria very motile.	At end of 20 hrs. partly dead. Cells full of vacuoles. Bacteria increased and motile.
21	" "	" "			" "	" "	" "
22	" "	" "			" "	" "	" "
23.	" "	" "			" "	" "	" "
24.	" "	No bacteria					At end of 20 hrs. good growth. Many mitotic figures. Very few vacuoles.
25.	I, 72 hrs.	12 hr. culture of No. 80 B M			Many vacuoles in all cells.		
26.	1, 48 hrs.	24 hr. culture of No. 80 B M.		Small vacuoles in all cells.	Many vacuoles. Fixed ⁹⁰ and stained.		

TABLE I—*Continued.*

Culture.	Bacteria.	Observations.				Later observations.
		After 10 min.	After 30 min.	After 1 hr.	After 2 hrs.	
27. 1, 48 hrs.	24 hr. culture of No. 80 B M.	Small vacuoles in all cells.	Many vacuoles.	Fixed and stained.		
28. "	"	"	"		Fixed and stained.	
29. "	"	"	"			
30. 1, 72 hrs.	60 hr. culture of No. 80 B M.	Few vacuoles.			A number of vacuoles in the cells. Clasmotocytes have taken up the bacteria.	At end of 3 hrs. fixed and stained.
31. "	Bacteria frequently long threads.	"	"		"	"
32. "	"	"	"		"	"
33. "	"	"	"		"	"
34. "	"	"	"		"	"

35. 1, 48 hrs.	24 hr. culture of No. 80 B M ₂ .	Few vacuoles.			At end of 4 hrs. stained with neu- tral red in Locke- Lewis solution. Many various shaped and sized red vacuoles, some containing one or more bacteria. Clasmotocytes have red vacu- oles containing unstained bac- teria. At end of 20 hrs. almost all growth dead. Some cells have un- stained vacuoles containing active bacteria. A few cells had red vac- uoles.
36. " " " (3 cultures).	No bacteria.	No " "			Lived 7 days. Good growth. Few vacuoles.

TABLE I—Continued.

Culture.	Bacteria.	Observations.			
		After 10 min.	After 30 min.	After 1 hr.	After 2 hrs.
37. I ₃ 48 hrs. Culture washed with Locke-Lewis solution + neutral red.	24 hr. culture of No. 80 B M.		Few vacuoles.		Many red vacuoles. No bacteria in vacuoles even in clasmotocytes.
38. " "	" "		" "		At end of 20 hrs. growth largely dead; diffuse red. Vacuoles unstained. A few cells still alive have many large red vacuoles.
39. " "	24 hr. culture of No. 80 B S ₂ .		" "		" "
40. " "	" "		" "		" "
41. I ₃ 48 hrs.	" "		" "		At end of 4 hrs. stained with neutral red in Locke-Lewis solution. A number of red vacuoles. Some red vacuoles have unstained bacteria in them. Some bacteria in red vacuoles motile; some outlined with red in the cell.

42. I ₈ 48 hrs.	24 hr. culture of No. 80 B S ₂ .				At end of 4 hrs. stained with neu- tral red in Locke- Lewis solution. A number of red vacuoles. Some red vacuoles have unstained bacteria in them. Some bacteria in red vacuoles motile; some out- lined with red in the cell.
43. I ₈ 72 hrs.	" "				" "
44. " "	" "				" "
45. I ₈ 48 hrs.	" "			A number of vac- uoles in each cell.	At end of 3 hrs. stained with neu- tral red. A num- ber of red vacu- oles have quiet bacteria in them. Many red vacu- oles in each cell.
46. " "	No bacteria.				At end of 4 hrs. no vacuoles. Lived 7 days. At end of this time there were a num- ber of vacuoles in some cells.

TABLE I—Continued.

Culture.	Bacteria.	Observations.			
		After 10 min.	After 30 min.	After 1 hr.	After 2 hrs.
47. I, 48 hrs.	24 hr. culture of No. 80 B S ₄ .		A number of vacuoles.		
48. " "	" "		A few vacuoles in each cell.	No increase in vacuoles.	At end of 20 hrs. cells almost all dead. Bacteria grew and are motile.
49. " "	" "		A number of vacuoles in each cell.		At end of 20 hrs. good growth. Many vacuoles in each cell. Bacteria increased and are motile.
50. " "	No bacteria.				At end of 20 hrs. all dead. Bacteria grew and are motile.
51. " "	24 hr. culture of No. 80 B S ₈ .				At end of 20 hrs. good growth. Many figures of division. A few vacuoles in a few cells.
52. " "	" "			Many vacuoles; some large.	An occasional vacuole.
			A number of vacuoles.		
			A number of vacuoles. Some dead cells.	Many vacuoles; some large. Fixed and stained.	

53. 1, 48 hrs.	24 hr. culture of No. 80 B S ₄ .	Many vacuoles.	Many vacuoles. Some large ones have one motile bacillus.	At end of 20 hrs. few cells survive; all full of vacuoles. Only one vacuole seen containing a bacillus.
54. " "	" "	No vacuoles.		At end of 20 hrs. no vacuoles.
55. " "	24 hr. culture of No. 80 C.	Many vacuoles. Many dead cells.	Many vacuoles. No bacteria seen in vacuoles.	At end of 20 hrs. all dead. Bacteria grew.
56. " "	" "	Many vacuoles. No dead cells.		At end of 20 hrs. a few spindle cells survive. These are full of vacuoles. Bacteria grew.
57. " "	" "	" "		Dead at end of 20 hrs. Bacteria grew.
58. " "	" "	Many vacuoles; some large.		" "
59. " " Washed with neutral red in Locke-Lewis solution.	" "	A few small red vacuoles.	A number of small red vacuoles.	At end of 20 hrs. all cells dead. Bacteria grew and are motile.
60. " "	" "	" "	" "	" "

TABLE I—*Concluded.*

Culture.	Bacteria.	Observations.			
		After 10 min.	After 30 min.	After 1 hr.	After 2 hrs.
61. 1-48 hrs. Washed with neutral red in Locke-Lewis solution.	24 hr. culture of No. 80 C.				Later observations. At end of 4 hrs. many red vacu- oles of different shapes, some con- taining an un- stained granule. Dead at end of 20 hrs.
62. " "	" "			A number of small red vacuoles.	At end of 4 hrs. many cells dying. A number of un- stained vacuoles. Many red vacu- oles. No bac- teria seen in vac- uoles. At end of 20 hrs. cytoplasm of cells has become a network and is full of unstained vacuoles. Cell is diffuse red. Bac- teria grew and are motile.
63. " "	" "			" "	" "

64. J, 48 hrs. Washed with neutral red in Locke-Lewis solution.	48 hr. culture of No. 80 B S ₄ .		A number of small red vacuoles.	At end of 20 hrs. a number of cells still alive; full of bright red vacu- oles. Bacteria motile.
65. " "	" "		" "	Dead at end of 20 hrs. Bacteria motile.
66. " "	" "		" "	At end of 20 hrs. some of growth still alive with cells full of large red vacuoles.
67. I, 24 hrs.	48 hr. culture of No. 80 B S ₆ .		Many vacuoles.	
68. " "	" "		" "	
69. " "	" "		" "	
70. " "	No bacteria.		No	At end of 20 hrs. good growth. Many mitotic figures.

regard to the effect of bacteria upon plasma cultures. He states that *Bacillus typhosus* never grew in plasma alone. However, when the plasma had been incubated to destroy its bactericidal action, the bacteria developed freely with especial affinity for the tissue cells. According to Smyth the typhoid bacteria had no toxic action on the tissue cells. It is difficult to understand this statement in the light of the present experiments, as in every culture in which the organism grew, there resulted a rapid degeneration of the tissue. It is possible that the strain of typhoid bacillus employed by Smyth was different from that used in these experiments.

The normal cultures contained many cells in the process of division. When these cultures were inoculated, the cells undergoing mitosis, as well as the resting cells, became vacuolated. The dividing cells completed the process but no new mitotic figures appeared in the growth, except in rare instances. While the normal culture of the series usually exhibited eight to ten dividing cells, even when the culture was more than 4 days old, the infected cultures seldom showed any.

When the cultures were stained with neutral red before inoculation the vacuoles were red from their first appearance. This color faded upon the death of the cell. The vacuoles were stained at various times during their formation and always became bright red, regardless of whether they contained bacteria or not. The motility of the organism in the vacuoles usually decreased when the preparation was stained with neutral red.

These vacuoles cannot be regarded as the result of the ingestion of the bacteria, as they appeared very rapidly in all the cells at the same time, being small at first but later increasing in size and even fusing to form exceedingly large vacuoles. The bacteria were never found in the cells until some time after the vacuoles had appeared and then only in a few of the many hundred cells comprising the growth. Even when present they were not found in all the vacuoles of a cell but usually in only one. The organisms were very motile in the unstained vacuole, occasionally dashing from side to side, but more frequently circling rapidly round and round the boundary of the vacuole. If the vacuoles are the result of the ingestion of some substance by the cell, as has been claimed by some writers, one

would not expect to find any difference in results, whether the original hanging drop were used or had been replaced by a fresh one. However, the utilization of a fresh drop did markedly alter the results; so that, instead of vacuoles appearing at once and rapidly increasing in size, it was some time before they became noticeable in numbers. The results obtained in regard to phagocytosis will not be given here, as they are at present inseparable from many other complicated factors.

Metchnikoff, who not only performed many experiments himself but discussed the work of innumerable other investigators upon the subject of phagocytosis, gives only a meager description of vacuoles in cells other than protozoa. Metchnikoff and his followers seemed to take it for granted that the leucocyte contained digestive vacuoles; this, however, is not the case. Normal leucocytes do not contain vacuoles, although they rapidly become vacuolated under abnormal conditions. Plato³ claims that there are no bodies that stain with neutral red in the leucocytes of the freshly drawn blood. Miss R. Prigosen in a number of observations upon the blood of the hen and also upon human blood in this laboratory found no vacuoles of any kind in leucocytes immediately upon their withdrawal from the body. Frequently writers describe the ingestion of bacteria without mentioning vacuoles, and other authors, even with the use of neutral red, claim that the ingested particles stain with neutral red, but they often either fail to describe the stained vacuoles or definitely state that the vacuoles remain unstained.

Barber inoculated *Nitella*, *Saprolegnia*, and *Achyla* with *B. typhosus*. In nearly every instance the organism grew luxuriantly in the cells. Barber does not mention vacuoles in the cytoplasm other than the vacuole which was a definite structure of the cell. He states that the bacteria apparently met with no resistance in passing from the cell vacuole into the protoplasmic layer. It was not possible to find any harmful action of the protoplasm upon the bacteria. The cell finally died as a result of parasitism.

A number of writers discussing necrosis, degeneration, or old age frequently describe vacuolation of the tissue. Calkins⁴ mentions this in regard to paramecium. Dr. W. Gary told me of certain experiments on autolysis of protozoa in which vacuoles appeared in the cytoplasm of the organism. Miss Prigosen, in the studies previously mentioned, cites a number of cases in which lack of oxygen caused the formation of vacuoles in different sorts of cells. Symmers shows vacuolation of the cells in multiple non-inflammatory necrosis of the liver. Lewis (1919, *b*) describes the vacuolation of tissue cultures associated with degeneration of these growths.

³ Plato, p. 913.

⁴ Calkins, p. 127.

Many substances have been introduced into the hanging drop of tissue cultures in an effort to obtain information as to what factor in the life history of the cell is chiefly concerned in the formation of vacuoles. Food substances such as egg albumin, blood serum, dextrose, glycogen, aleuronat, etc., when added to the medium did not cause a marked or rapid increase in the number of vacuoles. Some of these substances caused a slight increase in the number of neutral red granules. Other substances such as phosphorus, carbon dioxide, urea, and ammonia caused all sorts of distortions of the cells, and often death ensued, but they failed to result in a constant vacuolation of the cytoplasm. As far as has been determined the effect of the typhoid bacteria upon the culture seems to be one that sets up some disturbance of the normal behavior of the cell in such a way as to result rapidly in a type of degeneration which is not uncommon in old cultures under usual conditions; *i.e.*, such a degeneration as has been described by Lewis (1919, *b*) in uninoculated cultures. It would be interesting to determine whether cloudy swelling common in acute infection is a form of vacuolation of the cells.

CONCLUSION.

The introduction of *Bacillus typhosus* into the hanging drop of a tissue culture of the intestine of a chick embryo leads to the rapid vacuolation of the cells of the tissues which comprise the growth of the cultures. The cells of the connective tissue, the endodermal membrane, the mesothelium, and also the clasmatocytes exhibit the ability to ingest *Bacillus typhosus* in the cultures.

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EXPLANATION OF PLATES.

PLATE 18.

FIG. 1. 48 hour growth of mesothelium from the intestine of an 8 day chick embryo, 2½ hours after inoculation with *B. typhosus*.

FIG. 2. 48 hour culture of the intestine of an 8 day chick embryo. Vacuolated endodermal membrane due to the presence of *B. typhosus* in the hanging drop for 2½ hours.

PLATE 19.

FIG. 3. 48 hour culture of the intestine of an 8 day chick embryo. Bacilli were moving rapidly in a vacuole in the elongated binucleate cell near the center of the field. The other organisms seen in the photograph became fastened to the growth upon fixation. They were not within the living cells but moved freely in and out of the field of vision. The cells show the vacuoles arranged around the centrosphere at one side of the nucleus as described by Lewis (1919, *a*).

FIG. 4. 48 hour growth of the connective tissue from the intestine of an 8 day chick embryo after inoculation with *B. typhosus*. 1 hour after the introduction of the organism the cells were full of small vacuoles. These later became larger and fewer in number.

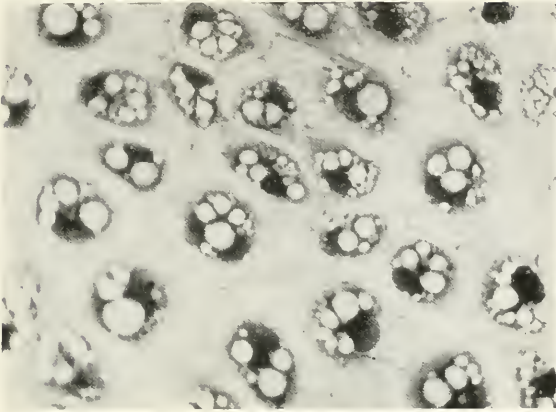


FIG. 1

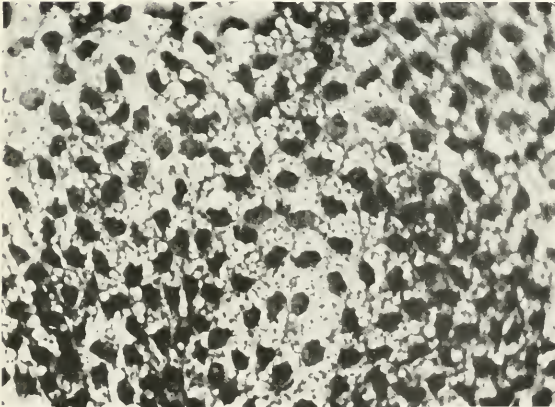


FIG. 2.

(Lewis; Vacuoles in cells of tissue cultures.)

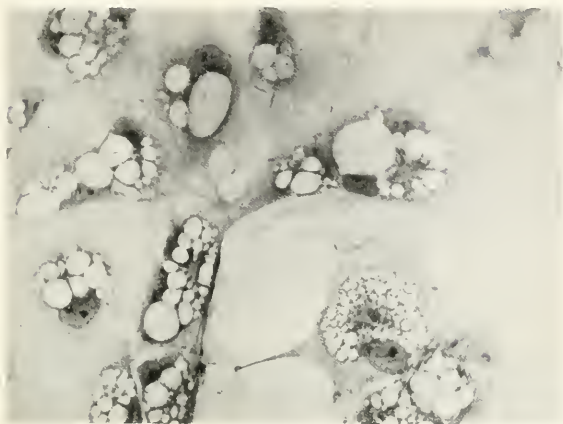


FIG. 3.

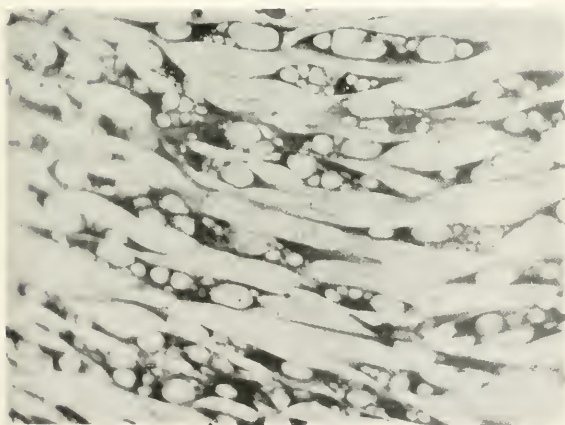


FIG. 4.

(Lewis: Vacuoles in cells of tissue cultures.)

APPEARANCE OF ISOAGGLUTININS IN INFANTS AND CHILDREN.

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The presence of isoagglutinins in human blood, first described by Shattock and Landsteiner (1900, 1901) has attracted a great deal of attention, especially since the introduction of blood transfusion and the recognition of reactions that may follow in some instances. At the suggestion of Dr. W. L. Moss, I undertook in 1914 to determine whether isoagglutinins, as present in the blood of adults, are present at birth, and if not, at what period of life they are established.

The presence of isoagglutinins in the blood of adults regardless of health or disease has been well established, and it is not intended here to state more than the fundamental facts of adult isoagglutination before passing to the question of infants.

Landsteiner (1900, 1901) noted that when the serum of several individuals was tested against the corpuscles of the same individuals, in a certain proportion clumping of the corpuscles, or isoagglutination, occurred.

Shattock at the same time (1900) noticed that when the serum of patients with pneumonia, erysipelas, typhoid, and acute rheumatism was added to normal human blood, clumping of the corpuscles, or agglutination, occurred. This he considered a pathological phenomenon since it was not found when normal human blood serum was added to normal blood. Donath found that the serum of patients with various blood diseases (chlorosis, secondary anemia, leucemia) agglutinated normal red blood corpuscles, whereas normal human sera would not agglutinate normal human red cells. Grünbaum observed that sera from cases of typhoid fever and scarlet fever would clump the corpuscles of a patient with another disease, but would not clump corpuscles from a patient with the same disease. Eisenberg found that the serum of normal individuals seldom (1 in 10) agglutinates the corpuscles of healthy individuals, but in a large series of patients ill with various diseases agglutination of the corpuscles frequently occurred. Lo

Monaco and Panichi noticed agglutination of the corpuscles in cases of malaria. Ascoli observed this reaction in both well and sick individuals, but in the latter in greater intensity. Landsteiner (1901) showed that the isoagglutination reaction occurred with normal blood, and divided individuals into three classes, according to the reaction, as follows:

Group A. Serum agglutinates corpuscles of Group B. Corpuscles are agglutinated by sera of Groups B and C.

Group B. Serum agglutinates corpuscles of Group A. Corpuscles are agglutinated by sera of Groups A and C.

Group C. Serum agglutinates corpuscles of Groups A and B. Corpuscles are not agglutinated by sera of either Group A or B.

Later, Langer, von Decastello and Sturli, Ascoli, and Hektoen confirmed the fact that the phenomenon of isoagglutination is independent of health or disease. Moss (1910) and Janský independently showed that the classification of Landsteiner was incomplete in that if the serum of a sufficiently large group of individuals is tested against the corpuscles of each of the group, some would be found who do not fall into any of the three groups of Landsteiner (Moss' Group I). Moss classified individuals as follows:

Group I. Serum agglutinates no corpuscles. Corpuscles are agglutinated by sera of Groups II, III, and IV.

Group II. Serum agglutinates corpuscles of Groups I and III. Corpuscles are agglutinated by sera of Groups III and IV.

Group III. Serum agglutinates corpuscles of Groups I and II. Corpuscles are agglutinated by sera of Groups II and IV.

Group IV. Serum agglutinates corpuscles of Groups I, II, and III. Corpuscles are agglutinated by no sera.

These relations may also be tabulated as follows:

Serum.	Corpuscles.			
	Group I.	Group II.	Group III.	Group IV.
Group I.	0	0	0	0
“ II.	+	0	+	0
“ III.	+	+	0	0
“ IV.	+	+	+	0

Moss proposed the following hypothesis as a probable explanation of the various isoagglutinin groups.

Group I. Serum contains no agglutinin. Corpuscles possess receptors a, b, and c.

Group II. Serum contains agglutinin A. Corpuscles possess receptors b and c.

Group III. Serum contains agglutinin B. Corpuscles possess receptors a and c.

Group IV. Serum contains agglutinin C. Corpuscles possess no receptors.

Von Dungern and Hirschfeld (1909-10, 1910, *b*, 1910-11) working at the same time found that individuals fall into four groups which they designated as follows:

- Group AB, corresponding to Moss' Group I.
- Group A, corresponding to Moss' Group II.
- Group B, corresponding to Moss' Group III.
- Group O, corresponding to Moss' Group IV.

They assumed that the agglutination reactions are due to the presence of two agglutinin radicals, A and B, in the corpuscles, and two agglutinins, α and β , in the serum, and explained the presence of the phenomena on the following basis.

Group AB (5.7 per cent) contains in the serum no agglutinin; contains in the corpuscles receptors A and B.

Group A (47.3 per cent) contains in the serum agglutinin β ; contains in the corpuscles receptor A.

Group B (11 per cent) contains in the serum agglutinin α ; contains in the corpuscles receptor B.

Group O (36 per cent) contains in the serum agglutinins α and β ; contains in the corpuscles no receptors.

Therefore, the serum of Group A agglutinates the corpuscles of Groups B and AB because they both contain the receptor B. The serum of Group B agglutinates the corpuscles of Groups A and AB, both containing the receptor A. The serum of Group AB agglutinates no corpuscles as the serum has no agglutinin. The serum of Group O agglutinates corpuscles of Groups A, B, and AB, as they contain receptor A or B, or both. These authors believe that the characters A and B are inherited according to the Mendelian law of heredity. Epstein and Ottenberg have also advanced this theory.

It will be seen that there are at least two, possibly more, explanations to account for the interagglutination reactions of human serum and corpuscles.

Various authors report the presence of isoagglutinins in animals (dogs, cats, steers, monkeys, etc. (Kahn and Ottenberg; Ottenberg, Friedman, and Kaliski; von Dungern and Hirschfeld (1909-10, 1910, *a*); Ingebrigtsen; Fishbein), but have failed to show that groups exist within an animal species as in the case of man.

As to the mechanism of production of isoagglutinins in the blood of man no definite proof has been forthcoming, although several explanations have been offered; *i.e.*, that isoagglutinins pass through the chorion from mother to fetus, that they are formed in infancy by an immunity reaction like bacterial agglutinins, that they are absorbed through the milk of the mother by the infant, that they are formed as the result of an immunity reaction against bacteria of the intestinal tract, and finally, that they are formed by the disintegration of red corpuscles in the individual (Halban, Ascoli, Eisenberg, Langer).

Although the isoagglutination reaction may be obtained with the blood of all adults, this is not universally true of infants. Halban was the first to study the isoagglutination reaction of maternal and fetal blood (blood from the umbilical cord at birth). He found that the agglutinating power of maternal serum was

greater than that of fetal serum as tested against adult and fetal corpuscles, and that maternal corpuscles were more readily agglutinated than were fetal corpuscles. He states that agglutinins do not necessarily pass from mother to fetus as the mother's blood may possess agglutinins which may be absent in the infant's blood.

Halban and Landsteiner found that mother's blood possessed a greater content of isoagglutinins as tested against rabbit blood than does infant's blood. Langer found that the serum of the newly-born agglutinated corpuscles of the mother in only three of eleven cases tested and seldom agglutinated adult corpuscles or corpuscles of other newly-born; he also found that the corpuscles of blood from the cord and from the newly-born were more often agglutinated by the serum of the newly-born and adults than were adult corpuscles by the serum of the newly-born, and finally, that the mother might possess isoagglutinins which were absent in the newly-born, which shows that isoagglutinins do not pass from the mother through the chorion to the fetus.

Von Decastello and Sturli tested the serum of newly-born infants (umbilical cord) against the corpuscles of the mother and of other adults, and found in a small series of seven tested that the blood of infant and mother may give different agglutination reactions. They noted further that the serum of the newly-born is usually weaker in agglutinin than that of the adult, and that agglutination is occasionally absent. In a series of eleven infants, 7 days to 4 months old, they noted frequently the absence of agglutinins in the serum, and concluded that with most young children the reaction is not so clear-cut as with adults. They state that serum soon after birth acquires agglutinin, and later the corpuscles themselves acquire agglutinoophilic receptors by an immunization process.

Von Graff and von Zubrzycki found that serum of blood from the umbilical cord is poorer in agglutinin against mother's corpuscles than is the mother's serum against corpuscles of other adults. Schenk (1904) tested the interagglutination reactions between mother's and infant's blood, and concluded that the sera of mothers seldom agglutinated corpuscles of their own children, and that the serum of the child is inactive against the corpuscles of the mother. In other words, the sera of the one and corpuscles of the other behave as if they belonged to the same individual. Recently Cherry and Langrock tested the blood of thirty infants against the mothers' blood and found no agglutination, and so concluded that one can safely use mother's blood for the transfusion of her own infant. In summary, then, it has been noted that blood from the fetal cord and the blood of newly-born infants is poorer in isoagglutinin than that of adults, and that the isoagglutinin is frequently absent.

In view of the uncertainty regarding isoagglutination of infants it was undertaken to determine (1) whether the isoagglutination groups as they occur in adults are present at birth; (2) if not present at birth, how soon after birth they are established; and (3) the order in which

the serum acquires agglutinins for the four adult groups of corpuscles, and the order in which the corpuscles become agglutinable by the four adult groups of sera.

Method.

In order to determine whether isoagglutinin was present in the sera of the infants studied, they were tested against the corpuscles of the four adult groups, and similarly, to determine whether the corpuscles of the infants were agglutinable they were tested against sera of the four adult groups. This procedure possessed the advantage of showing whether the serum or corpuscles of the infant fell into any of the established four adult groups. Serum and corpuscles were freshly obtained from infants and adults each time the tests were made. A large series of adults was therefore grouped in order to have available at any time the fresh serum and corpuscles of the four groups.¹

With a Wright tube a small amount of blood was drawn from the finger of the individual (adult) to be tested, sealed, allowed to clot, and centrifuged. One drop of this serum was placed on a cover-slip with a glass capillary pipette with one drop of a dilute suspension of washed red blood corpuscles of a known Group II, stirred, and inverted on a hollow ground slide. Similarly a drop of serum was placed on another cover-slip with a drop of a dilute suspension of washed red blood corpuscles of a known Group III, incubated for 1 hour, and read microscopically for agglutination. In this way, a large number of ward patients and laboratory workers were grouped. The following proportion of groups in several hundred adults tested was found.

	<i>per cent</i>
Group I.....	3
Group II.....	35
Group III.....	12
Group IV.....	50
	<hr/> 100

The serum and corpuscles were then obtained from each of the four adult groups—the serum in Wright tubes, which were centrifuged and the serum separated; the corpuscles collected in a tube containing

¹ In this paper the numbers of the groups are those used by Moss (1910).

1.5 per cent sodium citrate in 0.85 per cent sodium chloride and washed twice in 0.85 per cent sodium chloride. The serum and corpuscles were obtained fresh each time the tests were to be made. In a similar manner serum and corpuscles were obtained from a number of infants. To a drop of each of the four adult group corpuscles a drop of infant's serum was added. Similarly, to a drop of each of the four adult group sera a drop of infant's corpuscles was added. This may be represented diagrammatically as follows:

Corpuscles.				
	Group I.	Group II.	Group III.	Group IV.
Infant serum				

Sera.				
	Group I.	Group II.	Group III.	Group IV.
Infant corpuscles				

This makes eight tests for each infant's blood. The preparations were examined immediately to see whether the cells were evenly distributed and no false clumping was present, as may occur from insufficient washing or shaking, and were then incubated at 37.5°C. and read at the end of $\frac{1}{2}$, 1, and 2 hours.² The microscopic method was used throughout. It was found to be more accurate than the macroscopic for the reading of slight degrees of agglutination. By referring to the tabulation on page 314 the group of the unknown serum or corpuscles can be read.

RESULTS.

Fetal blood, that is blood taken from the umbilical cord at birth, was obtained first, and then the blood of infants and older children. The results of the reaction as tested against adult group corpuscles and sera are shown in Tables I to V.

² Great care was taken to procure clean glassware. The hollow ground slides were washed in boiling water, dried, placed in 95 per cent alcohol, and dried as used. The cover-slips were boiled, put in 95 per cent alcohol, and wiped clean as used. The glass capillary pipettes and centrifuge tubes for collecting red blood corpuscles were sterilized by dry heat. The salt and citrate solutions used were sterile.

TABLE I.

Blood from the Umbilical Cord and Infants up to 1 Month of Age.

No. of infant.	Race.	Sex.	Date.	Age.	Infant's serum versus corpuscles of.				Infant's serum group.	Infant's corpuscles versus serum of.				Infant's corpuscles group.	Infant's group serum and corpuscles.
					Group I.	Group II.	Group III.	Group IV.		Group I.	Group II.	Group III.	Group IV.		
			1914	days											
1	White.	M.	June 4	11	0*	0	0	0	I	0	+	+	+	I	I
2	Negro.	F.	" 4	4	0	0	0	0		0	+	0	+	III	
			" 9	9	0	0	0	0		0	+	0	+	III	
3	"	"	" 4	10	0	0	0	0		0	0	0	0		
4	White.	M.	" 4	1	0	0	0	0		0	0	+	+	II	
			" 9	6	0	0	0	0		0	0	+	+	II	
5	"	F.	" 4	2	0	0	0	0		0	0	+	+	II	
			" 15	13	0	0	0	0	I	0	+	+	+	I	I
6	"	"	" 4	5	+	+	0	0	III	0	0	0	0		
			" 6	7	+	+	0	0	III	0	0	0	0		
7	"	M.	" 4	F.B.†	0	0	0	0		0	0	0	0		
			" 6	2	0	0	0	0		0	0	0	0		
			" 16	12	+‡	0	0	0		0	0	0	0		
8	"	F.	" 5	F.B.	0	0	0	0		0	0	+	+	II	
			" 18	13	0	0	0	0	I	0	+	+	+	I	I
9	Negro.	"	" 6	F.B.	0	0	0	0		0	0	+	+	II	
			" 9	3	0	0	0	0		0	0	+	+	II	
			" 16	10	0	0	0	0	I	0	+	+	+	I	I
10	White.	"	" 6	F.B.	0	0	0	0		0	0	0	0		
11	"	M.	" 6	7	0	0	0	0		0	0	0	0		
12	Negro.	F.	" 6	7	+	+	+	+		0	0	0	+		
			" 16	17	0	0	0	0		0	0	+	+	II	
13	White.	M.	" 6	10	0	0	0	0		0	0	0	+ ^w		
14	"	"	" 7	F.B.	0	0	0	0		0	0	+	+	II	
			" 16	9	0	0	0	0	I	0	+	+	+	I	I
15	"	"	" 8	F.B.	0	0	0	0		0	0	0	+		
			" 18	10	0	0	0	0		0	+	0	+	III	
16	Negro.	F.	" 9	F.B.	0	0	0	0		+	+	+	+		
			" 18	10	0	0	0	0		0	0	+	+	II	
17	White.	"	" 9	3	0	0	0	0		0	0	+	+	II	
18	"	"	" 9	2	0	0	0	0		0	0	0	0		
			" 18	11	+ ^w	0	0	0		0	0	0	0		

* In the tables + indicates definite agglutination; +[?], doubtfully positive; +^w, weakly positive; 0[?], doubtful or very slight; 0, no agglutination.

† F.B. (fetal blood) is blood obtained from the umbilical cord at birth.

‡ Test not done.

TABLE I—*Concluded.*

No. of infant.	Race.	Sex.	Date.	Age.	Infant's serum versus corpuscles of.				Infant's serum group.	Infant's corpuscles versus serum of.				Infant's corpuscles group.	Infant's group serum and corpuscles.
					Group I.	Group II.	Group III.	Group IV.		Group I.	Group II.	Group III.	Group IV.		
			1914	days											
19	Negro.	M.	June 9	5	0	0	0	0		0	0	0	0		
	"	"	" 16	12	0	0	0	0		0	0	0	0		
20	"	F.	" 10	F.B.	0	0	0	0		0	0	+	+	II	
	"	"	" 18	9	+	0	0	0		0	0	+	+	II	
21	"	F.	" 11	F.B.	0	0	0	0		0	+	0 ²	+	III ²	
22	White.	"	" 13	"	0	0	0	0		0	0	+	+	II	
	"	"	" 18	5	0	0	0	0	I	0	+	+	+	I	I
23	"	M.	" 16	F.B.	0	0	0	0	I	0	+	+	+	I	I
24	"	F.	" 16	"	0	0	0	0		0	0	+	+	II	
25	Negro.	M.	" 16	"	0	0	0	0		0	0	+	+	II	
26	White.	F.	" 18	"	0	0	0	0		0	0	0	0		
27	"	M.	" 16	"	0	0	0	0		0	0	+	+	II	
			1915												
28	"	F.	Mar. 8	12	0	0	0	0		0	0	0	0		
29	"	"	" 8	6	0	0	0	0		0	0	+	+	II	
30	"	M.	" 8	9	0	0	0	0		0	0	+	+	II	
31	"	F.	" 8	5	0	0	0	0		0	0	+	+	II	
32	"	"	" 12	4	0	0	0	0		0	+	0	+	III	
33	Negro.	M.	" 12	6	0	0	0	0		0	0	+	+	II	
34	White.	F.	" 12	3	0	0	0	0		0	0	+	+	II	
35	"	M.	Apr. 15	21	0	0	0	0		0	0	+	+	II	
36	"	"	" 24	21	0	0	0	0		0	0 ²	0	+	III ²	
			1916												
37	"	"	Feb. 17	11	0	0	0	0		0	0	0	0		
38	"	"	May 25	8	+	+	+	0	IV	0	0	0	0	IV	IV
39	"	F.	" 16	12	+	0	+	0		0	0	0	0		
40	"	"	" 16	10	0	0	0	0		0	0	+	+	II	
41	"	"	" 16	11	+	0	0	0		0	0	+	+	II	
42	"	M.	" 16	20	+	0	0	0		0	0	0	0		
43	"	F.	" 16	16	+	0	0	0		0	0	0	0		
44	Negro.	"	" 25	2	0	0	0	0		0	0	0	0		
45	"	"	" 25	3	+	0	0	0		0	0	0	0		
46	White.	M.	" 25	12	0	0	0	0		0	0	0	0		
47	"	"	" 25	7	0	0	0	0		0	0	0	+		
48	Negro.	F.	" 25	5	0	0	0	0		0	+	0	+	III	
49	White.	M.	" 16	7	0	0	0	0		0	0	+	+	II	
	"	"	" 25	16	0	0	0	0		0	0	+	+	II	

In Table I is shown the results of 67 tests on 49 infants, the blood being obtained from birth up to the age of 1 month. The striking points may be summarized as follows: (1) At birth the group is es-

TABLE II.
Blood from Infants 1 to 3 Months of Age.

No. of infant.	Race.	Sex.	Date.	Age.	Infant's serum versus corpuscles of.				Infant's serum group.	Infant's corpuscles versus serum of.				Infant's corpuscles group.	Infant's group serum and corpuscles.
					Group I.	Group II.	Group III.	Group IV.		Group I.	Group II.	Group III.	Group IV.		
			1914	mos.											
50	White.	M.	June 11	3	+	0	0	0		0	0	0	0		
51	Negro.	F.	" 11	1	+	0	0	0		0	+	0	+	III	
52	"	"	" 14	1	0	0	0	0		0	0	0	0		
53	White.	"	" 14	2	0	0	0	0		0	0	0	0		
54	"	"	" 18	1	0	0	0	0		0	0	0	0		
			1915	wks.											
55	Negro.	"	Feb. 17	7	+	+	+	0	IV	0	0	0	0	IV	IV
				mos.											
56	White.	"	" 17	2½	0	0	0	0		0	+	0	+	III	
57	"	"	" 17	2½	0	0	0	0	I	0	+	+	+	I	I
				wks.											
58	"	M.	" 20	10	0	0	0	0		0	0	+	+	II	
59	"	"	" 23	7	0	0	0	0		0	0	+	+	II	
			Mar. 11	9	0	0	0	0	I	0	+	+	+	I	I
60	Negro.	"	Feb. 23	7	0	0	0	0		0	0	+	+	II	
			1916	mos.											
61	White.	F.	Apr. 15	1½	0	0	0	0		0	0	0	0		
			May 5	2	0	0	0	0		0	0	0	0		
62	"	M.	Apr. 21	2	+ ^w	+ ^w	0	0	III	0	0	0	0		
63	"	F.	" 24	2½	+ ^w	0	+ ^w	0	II	0	0	+	+	II	II
64	Negro.	M.	" 25	2	+	0 ^p	+	0	II	0	0	0	0		
65	White.	F.	Dec. 6	2	0	0	0	0		0	0	+	+	II	
				wks.											
35	"	M.	May 6	6	0	0	0	0		0	0	+	+	II	
36	"	"	June 12	10	0	0	0	0		0	+	0	+	III	

tablished in very few instances, and the serum of a newly-born infant may contain no agglutinin and his corpuscles be inagglutinable. (2) The group was established in only one of the fifteen samples of blood obtained from the cord. (3) The corpuscles become agglutinable be-

TABLE III.

Blood from Infants 3 to 6 Months of Age.

No. of infant.	Race.	Sex.	Date.	Age.	Infant's serum versus corpuscles of.				Infant's serum group.	Infant's corpuscles versus serum of.				Infant's corpuscles group.	Infant's group serum and corpuscles.
					Group I.	Group II.	Group III.	Group IV.		Group I.	Group II.	Group III.	Group IV.		
			1914	mos.											
66	White.	M.	June 11	4	0	0	0	0		0	0	+	+	II	
67	Negro.	F.	" 11	3½	+	+	0	0	III	0	+	0	+	III	III
68	White.	M.	" 18	3½	0	0	0	0	I	0	+	+	+	I	I
			1915												
69	"	"	Jan. 27	5	+	0	+	0	II	0	0	0	0		
70	"	F.	" 27	4	0	0	0	0		0	0	0	0		
			Mar. 10	5½	0	0	0	0		0	+	0	+	III	
71	"	M.	Jan. 27	5½	0	0	0	0		0	0	0	0		
72	"	F.	" 28	3½	0	0	0	0		0	0	0	0		
73	"	M.	Feb. 6	5	+	+	+	0	IV	0	0	0	0	IV	IV
74	"	F.	" 20	5½	0	0	0	0		+	+	+	+		
			Mar. 6	6	0	0	0	0		0	0	0	0		
75	"	M.	Feb. 20	5	0	0	0	0		0	0	0	0		
76	Negro.	F.	" 27	5	+	0	+	0	II	0	0	+	+	II	II
77	White.	"	" 27	4	+	0	+	0	II	0	0	+	+	II	II
78	"	M.	" 27	4	0	0	0	0		0	+	0	+	III	
			Mar. 11	4½	0	0	0	0		0	+	0	+	III	
79	"	F.	Feb. 27	3	0	0	0	0	I	0	+	+	+	I	I
80	"	"	Mar. 3	5	+	+	+	?	IV?	0	0	0	0	IV?	IV?
			1916												
81	"	M.	Apr. 21	3	0	0	0	0		0	0	+	+	II	
82	"	F.	" 24	3	0	0	0	0		0	0	0	0		
			June 15	4¾	0	0	0	0		0	0	0	0		
83	"	M.	Apr. 18	3½	0	0	0	0		0	0	0	0		
84	Negro.	"	" 21	4½	0	0	0	0		0	0	0	0		
85	White.	"	" 15	3½	0	0	0	0		0	+	w	0	III	
			May 6	4¼	0	0	0	0		0	+	0	+	III	
			" 26	5	0	0	0	0		0	+	0	+	III	
			June 12	5½	0	0	0	0		0	+	0	+	III	
86	Negro.	F.	Apr. 24	4	0	0?	0	0		0	0	0	0		
			May 6	4½	0?	+	0?	0		0	0	0	0		
62	White.	M.	" 24	3	+	w	+	w	III	0	0	0	0		
			June 12	3¾	+	+	0	0	III	0	0	0	0		

‡ Test not done.

fore agglutinin is present in the serum. (4) In only seven of the 49 infants tested was agglutinin present in the serum, though the corpuscles were agglutinated in thirty-two cases. (5) Isoagglutination in

TABLE IV.
Blood from Infants 6 to 12 Months of Age.

No. of infant.	Race.	Sex.	Date.	Age.	Infant's serum versus corpuscles of.				Infant's serum group.	Infant's corpuscles versus serum of.				Infant's corpuscles group.	Infant's group serum and corpuscles.				
					Group I.	Group II.	Group III.	Group IV.		Group I.	Group II.	Group III.	Group IV.						
1914					mos.														
87	White.	M.	June 16	11	+	+	0	0	III	0	0	0	0						
88	"	F.	" 16	8	+	+	0	0	III	0	+	0	+	III	III				
89	"	M.	" 16	9	+	+	0	0	III	0	0	0	0						
90	Negro.	F.	" 16	10	0 ²	0	0	0	I	0	+	+	+	I	I				
91	"	M.	" 16	10	+	+	0	0	III	0	+	0	+	III	III				
92	White.	"	" 14	7	0	0	0	0	I	0	+	+	+	I	I				
93	"	"	" 18	8	0	0	0	0	I	0	+	+	+	I	I				
1915																			
94	"	"	Jan. 27	10	0	0	0	0		0	0	0	0						
95	"	F.	" 27	6	+	+	+	0	IV	0	0	0	0	IV	IV				
71	"	M.	Feb. 27	6½	0	0	0	0		0	0	+	+	II					
96	"	"	Jan. 28	6½	+	0	+	0	II	0	0	+	+	II	II				
			Mar. 6	7¾	+	0	+	0	II	0	0	+	+	II	II				
97	"	"	Jan. 28	6	0	0	0	0		0	0	+	+	II					
98	"	"	Mar. 5	10	+	+	0	0	III	0	+	0	+	III	III				
99	Negro.	"	" 5	10½	0	0	0	0		0	0	0	0						
1916																			
100	White.	F.	Apr. 15	6½	+ ^w	0	+	0	II	0	0	+ ^w	+ ^w	II	II				
			May 6	7¼	+	0	+	0	II	0	0	+ ^w	+	II	II				
101	"	"	Apr. 19	10	0	0	0	0		0	0	+	+	II					
102	"	M.	" 19	11	+	+	+	0	IV	0	0	0	0	IV	IV				
103	"	"	" 19	8½	+	+	+	0	IV	0	0	0	0	IV	IV				
104	"	F.	" 21	9	+	+	+	0	IV	0	0	0	0	IV	IV				
105	"	"	" 21	8	+	0	+ ^w	0	II	0	0	+	+	II	II				
106	"	"	May 6	7¼	0 ²	0	+ ^w	0		0	0	+	+	II					
			" 26	8	+ ^w	0	+	0	II	0	0	+	+	II	II				
36	"	M.	Sept. 12	6	+	+	0	0	III	0	+	0	+	III	III				
82	"	F.	" 12	7¾	+	+	0	0	III	0	+	0	+	III	III				

such a way as to allow classification of the infant in one of the fixed adult groups occurred in only eight of the 49 cases tested. Of these one belonged to Group IV, and the other seven to Group I. No mem-

TABLE V.
Blood from Children 1 to 10½ Years of Age.

No. of child.	Race.	Sex.	Date.	Age.	Child's serum versus corpuscles of.			Child's serum group.	Child's corpuscles versus serum of.			Child's corpuscles group.	Child's group serum and corpuscles.
					Group I.	Group II.	Group III.		Group I.	Group II.	Group III.		
107	White.	M.	¹⁹¹⁴ June 11	13 mos.	+	+	0	III	0	+	0	+	III
			¹⁹¹⁵ Feb. 2	21 "	+	+	0	III	0	+	0	+	III
108	Negro.	F.	¹⁹¹⁴ June 14	18 "	+	+	+	IV?	0	0	0	0	IV?
109	"	M.	" 18	2 yrs., 1 mo.	+	+	0	III	0	0	0	0	
110	White.	"	¹⁹¹⁵ Jan. 27	3 " 7 mos.	+	0	+	II	0	0	+	+	II
111	"	"	" 27	4 " 1 mo.	+	0	+	II	0	0	+	+	II
112	"	"	" 27	5 " 2 mos.	0	0	0	I	0	+	+	+	I
113	Negro.	F.	" 27	3 " 2 "	+	+	0	III	0	+	0	+	III
			Mar. 5	3 " 3 "	+	+	0	III	0	+	0	+	III
114	"	M.	Jan. 28	19 mos.	+	+	+	IV	0	0	0	0	IV
115	White.	"	Feb. 2	20 "	+	+	+	IV	0	0	0	0	IV
116	"	F.	" 2	10½ yrs.	+	+	0	III	0	+	0	+	III
117	"	"	" 2	5½ "	+	+	+	IV	0	0	0	0	IV
118	"	"	" 2	4½ "	+	0	+	II	0	0	+	+	II
119	"	"	" 4	8½ "	+	+	+	IV	0	0	0	0	IV
120	"	"	" 4	3 yrs., 8 mos.	+	+	0	III	0	+	0	+	III
121	"	M.	" 4	3 yrs.	+	+	+	IV	0	0	0	0	IV
122	"	"	" 5	2 yrs., 9 mos.	+	0	+	II	0	0	+	+	II
123	"	"	" 6	3 " 9 "	+	0	+	II	0	0	+	+	II
124	Negro.	"	" 6	2 yrs.	+	+	+	IV	0	0	0	0	IV
125	White.	"	" 20	2 yrs., 5 mos.	+	+	0	III	0	+	0	+	III
126	"	"	Mar. 3	17 mos.	+	+	+	IV	0	0	0	0	IV
127	"	"	¹⁹¹⁴ June 18	16½ "	0	0	0	I	0	+	+	+	I
128	"	"	¹⁹¹⁶ Apr. 24	12 "	+	+	+	IV	0	0	0	0	IV
129	"	F.	" 24	22 "	+	+	+	IV	0	0	0	0	IV
130	"	"	" 15	2 yrs.	+	0	+	II	0	0	+	+	II
131	Negro.	"	" 21	2 "	+	0	+	II	0	0	+	+	II
70	White.	"	May 2	19 mos.	+	+	0	III	0	+	0	+	III
80	"	"	" 2	19 "	+	+	+	IV	0	0	0	0	IV
68	"	M.	" 2	2 yrs., 2 mos.	0	0	0	I	0	+	+	+	I
87	"	"	" 2	2 " 10 "	+	+	+	IV	0	0	0	0	IV
92	"	"	" 2	2 " 5 "	0	0	0	I	0	+	+	+	I
58	"	"	" 2	17 mos.	+	0	+	II	0	0	+	+	II
75	"	"	" 2	19½ "	+	+	+	IV	0	0	0	0	IV

* Serum agglutinated corpuscles of all four adult groups. Serum heated to 56°C. for 30 minutes still agglutinated all four groups of corpuscles.

bers of Group II or III were present. (6) The corpuscles may be agglutinated by a serum of Group IV only, or by the serum of Groups II and IV, or III and IV, without the serum possessing any agglutinins, and an infant's corpuscles that are agglutinated by the serum of

TABLE VI.
Summary of Agglutination Reactions.

Table No.	No. of individuals tested.	Age.	Corpuscles agglutinated.		Serum contained agglutinin.		Serum contained agglutinin; corpuscles agglutinated; i.e., fixed group.		Cases in which group was established.
			Nn. of infants.	Per cent.	No. of infants.	Per cent.	No. of infants.	Per cent.	
I	49	Birth to 1 mo.	32	65.3	7	14.2	8	16.3	Group I, 7 " II, 0 " III, 0 " IV, 1
II	18	1-3 mos.	10	55.5	6	33.3	4	22.2	" I, 2 " II, 1 " III, 0 " IV, 1
III	22	3-6 "	10	45.4	8	36.3	7	31.8	" I, 2 " II, 2 " III, 1 " IV, 2
IV	23	6-12 "	15	65.2	15	65.2	16	69.5	" I, 3 " II, 4 " III, 5 " IV, 4
V	32	1-10½ yrs.	18	56.2	28	87.5	31	96.8	" I, 4 " II, 8 " III, 6 " IV, 13
Total...	144								

Groups III and IV may later acquire receptors for the serum of Group II, the infant thus becoming a member of Group I. (7) The serum may possess agglutinin against the corpuscles of Group I only, or Groups I and II, or Groups I and III, or in one case Groups I, II, and III (Group IV).

In Table II are seen the results of twenty tests on eighteen infants, 1 to 3 months old. In these, agglutination of the corpuscles took place in ten cases and agglutinin was found in the serum in six. The group was established in serum and corpuscles in only four of the eighteen cases.

In Table III thirty-one tests on twenty-two infants, 3 to 6 months of age, showed that agglutination of the corpuscles occurred in ten cases, and agglutinins in the serum in eight cases; the group was established in serum and corpuscles in seven of the twenty-two.

In Table IV twenty-six tests on twenty-three infants, 6 to 12 months of age, showed that agglutination of the corpuscles occurred in fifteen cases, and agglutinins were present in the serum in fifteen; the group was established in serum and corpuscles in sixteen cases.

Table V shows the results of thirty-four tests on thirty-two children, 1 to 10½ years of age. Agglutination of the corpuscles took place in eighteen cases, and agglutinins were present in the serum in twenty-eight; the group was established in serum and corpuscles in thirty-one of the thirty-two.

These results are summarized in Table VI.

Results of Successive Agglutination Tests on the Same Infant.

By referring to Tables I to V it will be noted that in thirty-eight cases the agglutination tests were repeated in the same infants' blood. In twenty-three of these there was no change in the grouping obtained. In fifteen the reaction had changed (Table VII).

These results may be summarized as follows:

Instances in which corpuscles showed no agglutination at first examination; later acquired receptors, becoming Group II.....	1
Instances in which corpuscles showed no agglutination at first examination; later acquired receptors, becoming Group III.....	4
Instances in which corpuscles showed receptors for Group II at first examination; later acquired receptors, becoming Group I.....	6
Instances in which serum showed no agglutinin at first examination; later acquired agglutinin, becoming Group II.....	2
Instances in which serum showed no agglutinin at first examination; later acquired agglutinin, becoming Group III.....	3
Instances in which serum showed no agglutinin at first examination; later acquired agglutinin, becoming Group IV.....	1
Instances in which serum showed agglutinin for Group III at first examination; later acquired agglutinin, becoming Group IV.....	1

TABLE VII.

Changes in Agglutination Reaction on Repeated Examination.

No. of infant.	Age.	Infant's serum group.	Infant's corpuscles group.	Infant's blood group.
5	2 days.	Not established.	II	Not established.
13	"	I	I	I
8	F. B.	Not established.	II	Not established.
	13 days.	I	I	I
9	F. B.	Not established.	II	Not established.
	4 days.	" "	II	" "
11	"	I	I	I
12	7 days.	Not established.	Not established.	Not established.
17	"	" "	II	" "
14	F. B.	" "	II	" "
	10 days.	I	I	I
15	F. B.	Not established.	Not established.	Not established.
	10 days.	" "	III	" "
22	F. B.	" "	II	" "
	5 days.	I	I	I
36	3 wks.	Not established.	Not established.	Not established.
10	"	" "	III	" "
	6 mos.	III	III	III
59	7 wks.	Not established.	II	Not established.
9	"	I	I	I
70	4 mos.	Not established.	Not established.	Not established.
	5½ "	" "	III	" "
19	"	III	III	III
82	3 "	Not established.	Not established.	Not established.
	4¾ "	" "	" "	" "
	7¼ "	III	III	III
75	5 "	Not established.	Not established.	Not established.
19	"	IV	IV	IV

TABLE VII — *Concluded.*

No. of infant.	Age.	Infant's serum group.	Infant's corpuscles group.	Infant's blood group.
87	11 mos.	III	Not established. IV	Not established. IV
	2 yrs.	IV		
	10 mos.			
58	10 wks.	Not established.	II	Not established. II
	17 mos.	II	II	
71	5½ "	Not established.	Not established. II	Not established. "
	6½ "	" "		

It is thus evident that corpuscles possessing no agglutinophilic receptors, *i.e.* not agglutinated by any sera, may acquire receptors, becoming members of Group II, III, or I, and that Group II corpuscles may change to Group I by further acquisition of receptors. This is also probably true for Group III corpuscles.

Similarly a serum containing no agglutinin, *i.e.* agglutinating no corpuscles, may acquire agglutinin for Group II, or for Group III, or for Group IV, and a serum containing agglutinin for Group III may change to Group IV by further acquisition of agglutinin. This is also probably true for Group II.

I have not seen a serum give up agglutinin which it has once acquired or corpuscles give up receptors, and do not believe that this occurs.

It is apparent, therefore, that an infant may be born with agglutinin in his blood, but it is rarely present in serum and corpuscles at birth so as to place him in a fixed group. He acquires further agglutinin first in his corpuscles and then in his serum and establishes his quota of group agglutinins usually by the end of the 1st year. After 1 year thirty-one of the thirty-two children had their group established, the one exception (No. 109, Table V) showing a Group III serum, with his corpuscles still inagglutinable. After 1 year the relative proportion of the fixed groups is approximately the same as with adults.

Relation of the Mother's Blood Grouping to That of the Infant.

In twenty-three cases the group of the mother was determined simultaneously with that of the infant (tested against the same group corpuscles and sera). The results are given in Table VIII.

In twenty-three cases the mothers' groups were as follows:

Group I.....	1
Group II.....	9
Group III.....	4
Group IV.....	9
<hr/>	
Total.....	23

The infants' groups were as follows:

Group not established.....	16
Group I.....	3
Group II.....	1
Group III.....	3
Group IV.....	0
<hr/>	
Total.....	23

In cases in which the infant's group was established the following results were obtained.

Instances in which blood of mother and infant were of same group...	1
Instances in which blood of mother and infant were of different groups	6
Instances in which mother's serum agglutinated the infant's corpuscles	11
Instances in which mother's corpuscles were agglutinated by the infant's serum.....	3

It has been suggested by Cherry and Langrock and others that it is safe to transfuse infants with their own mother's blood without preliminary tests. It would appear from the results given above that this is not a safe practice for the possibility of changes from agglutination is frequently present. For example, the mother may belong to Group II and the infant to Group III, or *vice versa*, or the mother to Group II and the infant to Group I. This may occur in an infant as early as the 4th day (No. 2, Table VIII). It would seem safer, therefore, to make preliminary tests on all infants.

TABLE VIII.

Relation of the Grouping in the Mother's Blood to That in the Infant's Blood.

No. of infant.	Mother's blood group.	Infant's age.	Infant's serum group.	Infant's corpuscles group.	Infant's blood group.
		<i>days</i>			
2	II*	4	Not established.	III	Not established.
		9	" "	III	" "
3	IV	10	" "	Not established.	" "
4	IV*	1	" "	II	" "
		6	" "	II	" "
5	II*	2	" "	II	" "
		13	I	I	I
6	IV	5	III	Not established.	Not established.
		7	III	" "	" "
7	IV	F.B.	Not established.	" "	" "
		2	" "	" "	" "
		12	" "	" "	" "
8	II*	F.B.	" "	II	" "
		13	I	I	I
11	IV	7	Not established.	Not established.	Not established.
13	IV	10	" "	" "	" "
		<i>mos.</i>			
70	III	4	" "	" "	" "
		5½	" "	III	" "
		19	III	III	III
96	III*†	7½	II	II	II
97	II	6	Not established.	II	Not established.
		<i>wks.</i>			
59	II*	7	" "	II	" "
		9	I	I	I
		<i>days</i>			
28	IV	12	Not established.	Not established.	Not established.
29	III*	6	" "	II	" "
30	II	9	" "	II	" "
31	II	5	" "	II	" "
32	III	4	" "	III	" "
33	IV*	6	" "	II	" "
34	IV*	3	" "	II	" "
		<i>wks.</i>			
36	II*†	3	" "	Not established.	" "
		10	" "	III	" "
		<i>mos.</i>			
		6	III	III	III
65	I	2	Not established.	II	Not established.
82	II*†	3	" "	Not established.	" "
		4½	" "	" "	" "
		7¾	III	III	III

* Infant's corpuscles agglutinated by mother's serum.

† Mother's corpuscles agglutinated by infant's serum.

Presence of Group Isoagglutinins in the Mother's Milk and Their Relation to the Infant's Blood Grouping.

As is well known, various bacteriological antibodies have been found to pass over from the mother's blood to mother's milk, and through the milk to her nursing infant, and subsequently to appear in the infant's blood. Ehrlich first demonstrated this by immunizing female mice to abrin, ricin, swine plague, diphtheria, and tetanus toxin, and demonstrating the presence of antitoxin to these substances in the milk of the mother and through the milk to the blood of the nursing young. Brieger and Ehrlich showed that this was also true of diphtheria antitoxin. Römer (1901) found that colts nursing mares immunized to diphtheria acquire considerable diphtheria antitoxin in the blood, and Römer and Sames, and Römer and Much showed that newly-born sheep acquire immunity to tetanus by nursing immune mothers or by having the milk of immune mothers fed *per os*, and the presence of tetanus antitoxin may be demonstrated in the blood. They explained this by the fact that the intestines of the newly-born animal are permeable to foreign protein (antitoxin) which passes through to the blood. After the 3rd day little is absorbed through the intestines. They found this to be true also in a calf during the first 6 days of life. They state that more antitoxin is absorbed through the colostrum than through the milk, as colostrum contains more albumin and globulin. A number of other authors have confirmed this.

Other substances, such as alexin (Moro) and hemolysins (Pfaundler and Moro), have been shown to appear in milk.

It was first demonstrated by Widal and Siccard (1897, *a, b*), later by Landouzy and Griffon, Castaigne, Römer (1909), and Rosenau and McCoy that typhoid agglutinins may pass from mother to nursing infant by way of the milk. In regard to hemagglutinins, Kraus by injecting rabbits with dog corpuscles found immune hemagglutinins in the blood and milk of rabbits, but though present in the milk they were not found in the blood of the nursing young rabbits. Langer first demonstrated isoagglutinins in the milk and colostrum of human mothers, but was unable to demonstrate them in the blood of ten nursing infants, 2 to 9 months of age, although present in mothers' milk. Schenk (1905) found that the milk of normal women contains isoagglutinin as does the colostrum. Von Zubrzycki and Wolfsgruber found normal isoagglutinins in human milk. These are present in greater proportion in the first 10 days of the puerperium than later, but they found the agglutinins present in small amounts or absent in the blood of nursing infants.

I was able to confirm this by examining the breast milk of fourteen nursing women, finding in each instance isoagglutinins present (Table IX). Furthermore, the isoagglutinins fell into groups identical with those of the mothers' blood. The groups were determined by testing the milk against corpuscles of Groups II and III, the method employed being similar to that used in testing the group of a serum.

In each instance the agglutination of the serum and corpuscles of the nursing infants was also tested. It will be seen that of the fourteen infants tested, in only five was the group established in the serum and corpuscles, although one (No. 97) had been nursing the mother for

TABLE IX.

Relation of the Grouping in the Mother's Milk to That in the Infant's Blood.

No. of infant.	Date.	Mother's blood group.	Mother's milk group.	Infant's age.	Infant's serum group.	Infant's corpuscles group.	Infant's blood group.
	1915			mos.			
96	Mar. 6	III	III	7 $\frac{3}{4}$	II	II	II
97	" 6	II	II	7 $\frac{1}{2}$	Not established.	II	Not established.
				days			
31	" 8	II	III	5	" "	II	" "
29	" 8	III	II	6	" "	II	" "
30	" 8	II	II	9	" "	II	" "
28	" 8	IV	IV	12	" "	Not established.	" "
				mos.			
70	" 10	III	III	5 $\frac{1}{2}$	" "	III	" "
	1916						
	May 2			19	III	III	III
				wks.			
59	Mar. 11	II	II	9	I	I	I
				days			
33	" 12	IV	IV	6	Not established.	II	Not established.
34	" 12	IV	IV	3	" "	II	" "
32	" 12	III	III	4	" "	III	" "
				wks.			
36	June 12	†	†	10	" "	III	" "
				mos.			
	Sept. 12	II	II	6	III	III	III
82	Apr. 24	†	†	3	Not established.	Not established.	Not established.
	Sept. 12	II	II	7 $\frac{3}{4}$	III	III	III
65	Dec. 6	I	I	2	Not established.	II	Not established.

† Test not done.

7 $\frac{1}{4}$ months. The blood of the fourteen nursing infants had no more isoagglutinin than did the blood of other infants, artificially fed, tested at the same age, so it would appear that the infant does not acquire his hemisoagglutinin through his mother's milk.

SUMMARY AND CONCLUSIONS.

1. The isoagglutination reaction of 131 infants and children from birth to $10\frac{1}{2}$ years was examined by testing their serum and washed corpuscles microscopically against the serum and corpuscles of each of the four adult groups.
2. The grouping as present in adults is rarely present in blood from the umbilical cord.
3. At birth and during the 1st month of life isoagglutination is rarely present, but the percentage of infants in whom the isoagglutinin group is established increases with age, so that after 1 year the group is usually established, and after 2 years is always present as in adults.
4. The grouping is established in the corpuscles before it is established in the serum; *i.e.*, the corpuscles acquire agglutinophilic receptors before the serum acquires agglutinin. Therefore, Group I is the first group to be formed and Group IV is the last.
5. The early grouping in the corpuscles before the group is established in the serum is liable to change by the acquisition of new receptors.
6. When the grouping has been established in both serum and corpuscles it does not change.
7. Isoagglutinins are present in mother's milk and the grouping is identical with that in the mother's blood. These agglutinins are probably not transmitted to the nursing infant through the milk.
8. On account of the differences between the agglutination reactions in the blood of mother and child it is not safe to transfuse an infant from its mother without making the preliminary tests.

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EFFECT OF FEEDING THE PINEAL BODY UPON THE DEVELOPMENT OF THE ALBINO RAT.

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PLATE 20.

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In a study during the past 2 years of the relation of certain endocrine glands to growth, two series of experiments were carried out with the purpose of ascertaining more accurately the effects of feeding with the pituitary gland and the thymus.¹ The following experiments were undertaken in the hope of adding further knowledge concerning the function of the pineal body, especially in its relation to growth and early sexual changes.

Method.

The method employed in our experiments consists in the oral administration of desiccated pineal powder.² The pineal body of young calves was minced, dried *in vacuo* at a temperature of 35–40°C., and extracted with ether. 1 gm. of this fat-free desiccated pineal substance represents 7 gm. of the fresh pineal body. The powder was given once or twice daily in doses varying from 0.01 to 0.1 gm. It was mixed with a small amount of bread and milk and the mixture was given to the rats before they received their daily rations. The animals were placed in small compartments when fed the powder, in order to insure its complete ingestion.

The albino rat (*Mus norvegicus albinus*) of the standard stock of the Wistar Colony, Philadelphia, was selected as the animal most suitable for our experimental work. This rat undergoes striking developmental changes in a relatively short period. Moreover, exten-

¹ Work not yet published.

² This powder was obtained from Armour and Company, Chicago.

sive studies of its normal development have been made by Donaldson,³ whose statistics we have used for comparative study.

The experiments were begun when the rats were about 3 weeks of age, immediately after weaning. The young animals were placed in pairs, in separate compartments of specially constructed cages. Precautions were taken to secure ideal hygienic surroundings, to give protection against light and noise, and to afford ample room for the normal activities of the animals. The diet of the rats consisted of wheat, corn, bread, and milk. In the first two experiments the animals were given an excess of bread and milk and mixed grains twice daily. In the third experiment each animal was given equal weighed amounts of Graham flour and milk. The rats were fed liberally and the amount of food was increased as the animals grew. In the last experiment equal amounts of bread and milk were given to all the animals, but no attempt was made to control the exact amount of grain. In this experiment an amount of desiccated thymus equal to the amount of pineal powder was given to two of the control animals. The other control animals were given a corresponding amount of purified casein.

Observations were made in regard to all gross developmental changes such as appearance and texture of the coat, descent of testes and their appearance at various ages, state of nutrition, skeletal changes, body weight, and activities of the animals (the last two recorded weekly). The rats were killed at intervals of 3, 5, and 6 weeks after the pineal feeding had been begun. At autopsy measurements of body and tail length were made, and tissue was obtained from the endocrine glands and the organs of reproduction. The tissue was placed in formaldehyde-Zenker's solution, made with neutral formaldehyde. The sections were stained with hematoxylin and eosin for microscopic study.

RESULTS.

During the entire series of experiments all the animals remained in an excellent state of nutrition. Although the powder of the pineal body was given in relatively large doses, it in no way affected their general health. The activities of the test animals equalled and cor-

³ Donaldson, H. H., *Memoirs of the Wistar Institute*, No. 6, Philadelphia, 1915.

responded to those of the control animals. In no instance was any sexual precocity noted. The testes of the pineal-fed rats and their control rats descended almost simultaneously. Furthermore, no differences were noted in the appearance, size, or consistency of the testes of test and control animals of the same litters.

The results are summarized in Tables I to VIII and graphically presented in Text-figs. 1 to 5. There was essentially no variation between the weight of the pineal-fed animals and their respective controls. The pineal-fed males showed a difference in weight of 0.5 per

TABLE I.

Gain in Weight of the Rats in Litter 1.

Born Dec. 22, 1916. Six animals. Feeding begun Jan. 9, 1917. Age 18 days. Animals killed Feb. 13. Duration of experiment 35 days.

0.01 gm. of pineal powder given twice daily until Jan. 16; 0.02 gm. twice daily thereafter. Pineal powder given in very small amount of food to insure its ingestion. All animals given excess of wheat bread, corn bread, and milk; occasionally cracked corn and oats. Animals were all extremely healthy and vigorous throughout experiment. Testes descended about Jan. 27—age 36 days.

Sex.	Animal.	Jan. 9.	Jan. 13.	Jan. 20.	Jan. 27.	Feb. 3.	Feb. 13.	Total gain.	Gain.
		gm.	gm.	gm.	gm.	gm.	gm.	gm.	per cent
M.	Control	20.0	25.0	38.0	55.0	66.5	90.0	70.0	350
F.	"	16.5	21.0	33.5	50.5	61.0	80.0	63.5	385
M.	Pineal-fed	17.5	22.0	32.0	46.0	58.0	75.5	58.0	331
F.	"	17.0	22.0	34.5	46.0	55.0	67.0	50.0	294
M.	"	18.5	22.5	34.5	50.0	56.5	75.5	57.0	308
F.	"	17.0	22.0	35.5	49.5	57.0	74.0	57.0	335

cent less than their control animals and the females 9.6 per cent less than their control animals. Not only was there essentially no difference in the final weights of the two sets of rats but no striking variation was noted at any stage of the period of observation. In Litters 1 and 2 it will be seen that the percentage of weight gained by the control rats is somewhat greater than that gained by the pineal-fed animals. This is not true of Litters 3 and 4. Comparisons of the weights of the testes of the pineal-fed rats and their control animals and of the respective ratios of testicular weight to body weight show no difference beyond what may be considered the normal coefficient of varia-

TABLE II.

Measurements and Weights of Organs of the Rats in Litter 1.

Sex.	Animal.	Body.	Tail.	Thymus.	Testes.	Ratio of testicular weight to body weight.
		cm.	cm.	gm.	gm.	per cent
M.	Control.....	15.5	12.9	0.312	1.447	1.60
F.	".....	15.0	11.2	0.355		
M.	Pineal-fed.....	14.9	11.9	0.205	1.412	1.87
F.	".....	14.5	11.6	0.200		
M.	".....	14.8	12.2	0.235	1.050	1.39
F.	".....	14.8	12.4	0.230		

TABLE III.

Gain in Weight of the Rats in Litter 2.

Born Dec. 25, 1916. Six animals. Feeding begun Jan. 13, 1917. Age 18 days. Animals killed Feb. 3. Duration of experiment 22 days.

0.01 gm. of same powder that was used for Litter 1 given daily until Jan. 16; 0.02 gm. twice daily thereafter. Diet as in Table I. Animals were all healthy and vigorous throughout experiment. Testes descended about Jan. 27—age 33 days.

Sex.	Animal.	Jan. 13.	Jan. 20.	Jan. 27.	Feb. 3.	Total gain.	Gain.
		gm.	gm.	gm.	gm.	gm.	per cent
M.	Pineal-fed.....	12.0	23.0	40.5	47.0	35.0	292
F.	".....	12.0	24.0	38.0	43.5	31.5	263
"	".....	12.5	26.5	38.5	46.0	33.5	268
"	".....	13.0	27.0	38.0	45.0	32.0	246
M.	Control.....	13.0	28.0	43.5	56.0	43.0	331
F.	".....	13.5	27.5	43.5	55.5	42.0	311

TABLE IV.

Measurements and Weights of Organs of the Rats in Litter 2.

Sex.	Animal.	Body.	Tail.	Thymus.	Testes.	Ratio of testicular weight to body weight.
		cm.	cm.	gm.	gm.	per cent
M.	Pineal-fed.....	12.2	10.2	0.156	0.469	0.998
F.	".....	11.5	10.4	0.115		
"	".....	11.9	10.1	0.120		
"	".....	11.4	10.8	0.110		
M.	Control.....	12.7	11.2	0.175	0.642	1.146
F.	".....	12.4	11.4	0.175		

TABLE V.

Gain in Weight of the Rats in Litter 3.

Born Feb. 1, 1917. Eight animals. Feeding begun Feb. 21. Age 21 days. Animals killed Apr. 5. Duration of experiment 43 days.

Fresh powdered desiccated pineal body from calves; first lot until Mar. 19, second lot until end of experiment. 0.0175 gm. of powder once daily until Feb. 24; 0.035 gm. once daily until Mar. 31; 0.035 gm. twice daily thereafter. Animals fed exactly equal amounts of milk and Graham flour. Food increased as the animals grew. Animals well throughout experiment, except for diarrhea which occurred intermittently during the experiment. This did not interfere with the vigor or playfulness of the animals. Testes descended about Mar. 18—age 46 days.

Sex.	Animal.	Feb. 21.	Feb. 27.	Mar. 6.	Mar. 13.	Mar. 24.	Mar. 31.	Apr. 5.	Total gain.	Gain.
		gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	per cent
M.	Control.	15.5	19.0	29.5	34.5	64.0	84.0	96.5	81.0	523
F.	"	14.5	17.5	28.0	33.0	57.5	76.5	86.0	71.5	493
M.	Pineal-fed.	15.0	17.0	27.0	33.0	62.0	85.5	98.5	83.5	557
F.	"	13.5	16.5	28.0	32.0	59.0	78.5	91.0	77.5	574
M.	"	14.5	17.5	29.0	33.5	62.5	85.5	102.5	88.0	607
F.	"	15.0	18.0	30.5	33.0	60.0	79.5	89.5	74.5	497
M.	Control.	14.5	17.5	29.0	32.5	61.5	83.0	100.5	86.0	593
F.	"	11.0	12.5	20.5	22.0	41.5	56.0	65.5	54.5	495

TABLE VI.

Measurements and Weights of Organs of the Rats in Litter 3.

Sex.	Animal.	Body.	Tail.	Thymus.	Testes.	Ratio of testicular weight to body weight.
		cm.	cm.	gm.	gm.	per cent
M.	Control.	16.3	13.2	0.260	1.840	1.907
F.	"	14.7	13.9	0.241		
M.	Pineal-fed.	15.7	13.7	0.240	1.465	1.487
F.	"	15.4	12.8	0.278		
M.	"	15.9	13.1	0.242	1.535	1.498
F.	"	15.3	13.8	0.274		
M.	Control.	15.9	12.7	0.268	1.345	1.338
F.	"	14.2	11.1	0.202		

tion. The total testicular weight of the pineal-fed rats is 10 per cent less than that of the control rats. The measurements of body and tail length yield results similar to the results obtained in regard to

TABLE VII.

Gain in Weight of the Rats in Litter 4.

Born Mar. 10, 1917. Six animals. Feeding begun Mar. 31. Age 21 days. Animals killed May 5. Duration of experiment 35 days.

Same pineal powder used as in last part of experiment with Litter 3. 0.05 gm. of powder given twice daily. Same thymus as in contemporary thymus experiments. Animals given equal amounts of bread and milk. Health good except for bad infection with lice in middle of experiment, from which the male control suffered the most. All cured by sulfur ointment. Testes descended about Apr. 9—age 30 days.

Sex.	Animal.	Mar. 31.	Apr. 7.	Apr. 14.	Apr. 21.	Apr. 28.	May 5.	Total gain.	Gain.
		gm.	gm.	gm.	gm.	gm.	gm.	gm.	per cent
M.	Control.....	15.0	23.5	33.5	46.5	67.0	80.0	65.0	433
F.	“.....	18.0	24.5	38.0	53.0	78.0	93.0	75.0	417
M.	Pineal-fed.....	18.0	25.5	40.5	62.0	92.0	113.0	95.0	528
F.	“.....	17.0	28.5	44.5	61.5	88.5	105.0	88.0	518
M.	Thymus-fed.....	16.5	26.0	44.0	62.0	88.5	111.0	94.5	573
F.	“.....	19.0	25.5	42.5	59.0	84.0	96.0	77.0	405

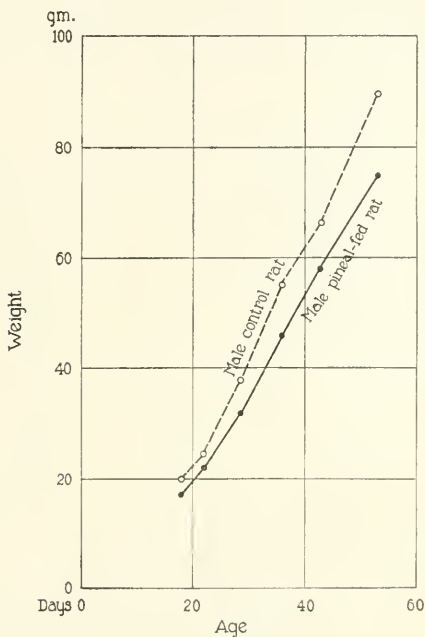
TABLE VIII.

Measurements and Weights of Organs of the Rats in Litter 4.

Sex.	Animal.	Body.	Tail	Thymus.	Testes.	Ratio of testicular weight to body weight.
		cm.	cm.	gm.	gm.	per cent
M.	Control.....	15.0	12.4	0.225	1.278	1.598
F.	“.....	15.5	14.3	0.240		
M.	Pineal-fed.....	16.8	14.3	0.330	1.525	1.349
F.	“.....	16.2	13.5	0.355		
M.	Thymus-fed.....	16.6	14.3	0.355	1.838	1.657
F.	“.....	15.9	14.2	0.255		

weight; namely, that the body and tail lengths of the control rats of Litters 1 and 2 are greater than those of the pineal-fed animals and that no noteworthy differences between members of Litters 3 and 4

are present, provided the comparison is limited to members of the same litters. Furthermore, the results of the study of body weights and measurements are in accord with the observations frequently made in regard to the size of the animals. These observations showed that

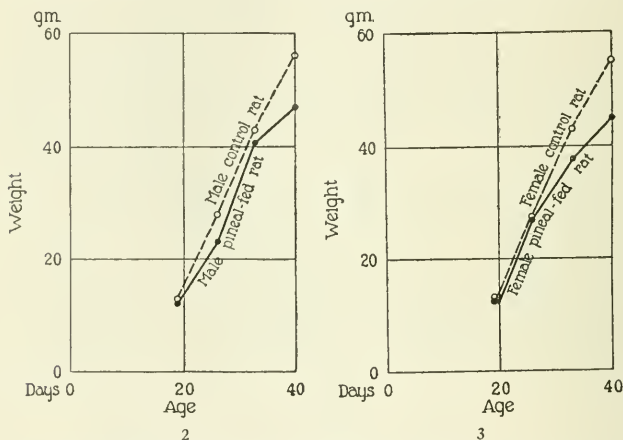


TEXT-FIG. 1. Curves showing the gain in weight of a male pineal-fed rat from Litter 1 and its control rat.

the difference in the size between the control and pineal-fed animals of the first two litters was slight, and no difference in members of the last two litters existed.

Microscopic studies of the endocrine glands of both control and pineal-fed animals revealed normal conditions; at least no differences

were discovered by the ordinary staining methods. Microscopic examination of the reproductive organs of the pineal-fed and control rats (male and female) likewise failed to reveal differences. All the results were entirely in accord with the normal findings in animals of the ages examined with the exception of the testes of the thymus-fed rats in Litter 4, which showed slightly more advanced spermatogenesis.



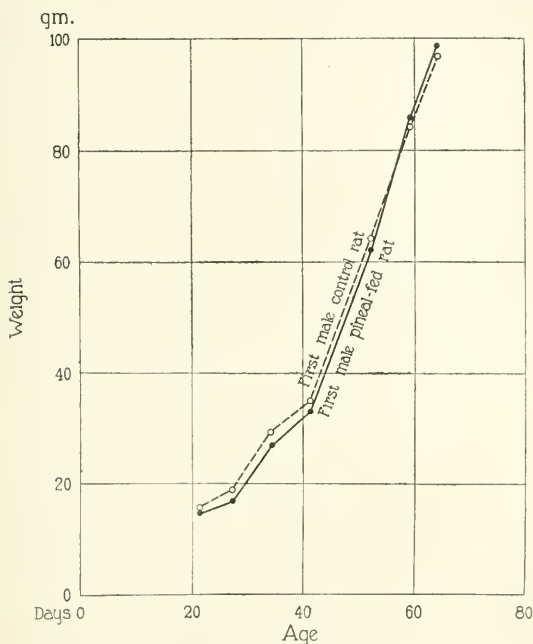
TEXT-FIGS. 2 and 3. Curves showing the gain in weight of a male pineal-fed and female pineal-fed rat from Litter 2 and their control rats.

DISCUSSION.

Observation of the pineal-fed and control rats for differences in activity and gross developmental change failed to suggest the existence of any active principle in the pineal body. The statistics in regard to weight and body measurement and the microscopic findings, which represent the most accurate criteria of developmental changes, also are confirmatory of the view that the pineal body does not possess an internal secretion. It will be noted that in Litter 4 the pineal-fed animals showed a greater variation in weight than can be explained by the normal variation of the species. We ascribe this variation to

the severe skin infection from which these animals suffered, rather than to any specific effect of the pineal feeding.

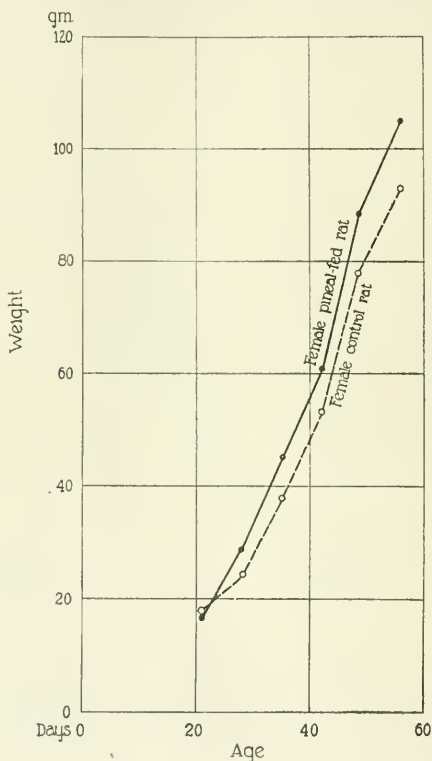
Special attention was given to any sexual changes that the pineal body might produce, because of the common belief that tumors of



TEXT-FIG. 4. Curves showing the gain in weight of a male pineal-fed rat from Litter 3 and its control rat.

this body cause sexual precocity and premature sexual development. Neither the activities of the test rats, nor the appearance, size, or weight of the testes, nor the time of their descent, nor the results of microscopic examination of either testes or ovaries suggested any relation between the pineal body and the reproductive organs.

There is little uniformity in the results obtained by previous workers who have studied the function of the pineal body. McCord⁴ has expressed the opinion that



TEXT-FIG. 5. Curves showing the gain in weight of a female pineal-fed rat from Litter 4 and its control rat.

the pineal body "contains some substance capable of stimulating growth." He based his conclusion upon a review of the literature and experiments in which

⁴ McCord, C. P., *Tr. Am. Gynec. Soc.*, 1917, xlii, 41.

either pineal extract was injected or the gland fed. Hoskins⁵ concluded that the pineal body, when fed to albino rats, has no effect upon their development. Other observers, using a different method of study, also have obtained conflicting results. Dandy⁶ removed the pineal body from young animals and was unable to note any developmental changes. Horrax,⁷ on the other hand, using a similar method of extirpation, arrived at the conclusion that the pineal body stimulated growth and sexual development. Fenger⁸ studied the physiological properties of the pineal body and found it essentially inactive. Park,⁹ who made a study of the literature, states: "It seems probable that a mass of experimental literature of conflicting nature will develop about the pineal body, like that which has sprung up around the thymus and the spleen. It can now be said that the experimental work on the pineal up to the present time has failed to prove that it possesses a function."

An accurate interpretation of feeding experiments with gland substances demands that certain factors which may explain the conflicting results of various observers should be considered. In a previous study on the effect of hypophyseal feeding on growth, attention was called to the importance of using standardized stock such as the albino rat used in the present experiments.¹⁰ As stated above, the normal development of this animal has been carefully studied and the statistics accumulated afford valuable data for comparison. The diet of the animal is another factor which may modify the results of feeding experiments. Little consideration has been given to the question of the adequacy of the diet in the past. We now know that not only adequate amounts of food elements are necessary but also that salts and so called food accessories have a direct relation to growth. It is important, therefore, that these substances should be given in equal amounts to control and test animals. Finally, mention should be made of the experimental method employed in its relation

⁵ Hoskins, E. R., *J. Exp. Zool.*, 1916, xxi, 295.

⁶ Dandy, W. E., *J. Exp. Med.*, 1915, xxii, 237.

⁷ Horrax, G., *Arch. Int. Med.*, 1916, xvii, 627.

⁸ Fenger, F., *J. Am. Med. Assn.*, 1916, lxvii, 1836.

⁹ Park, E. A., *Am. J. Dis. Child.*, 1916, xii, 477.

¹⁰ Work not yet published.

to the interpretation of the results. Oral administration of gland substances may be open to the criticism that the gland is broken down by digestive processes and thus becomes inactive. There is no evidence that such a change in the pineal body does or does not take place. The results of extirpation of the pineal body or injections of the gland may be modified by the trauma involved. The feeding method applied to the albino rat seemed to us the one most likely to reveal the true nature of pineal function.

SUMMARY AND CONCLUSION.

The pineal body of young calves was fed to albino rats beginning at the age of 3 weeks and extending over periods of from 3 to 6 weeks. Four litters of rats were used. Of these, fourteen rats were fed the pineal powder and ten were used as control subjects. Observations in regard to developmental changes and microscopic examination of the endocrine glands and reproductive organs were made. The pineal-fed rats of the first two litters remained somewhat smaller than their controls. The pineal-fed and control animals of Litters 3 and 4 showed no differences in development. Microscopic studies showed no differences between pineal-fed and control rats.

We may therefore conclude that feeding the desiccated pineal body of young calves to young albino rats fails to produce any effect upon the early development of these animals.

EXPLANATION OF PLATE 20.

FIG. 1. Photograph of (1) pineal-fed male, (2) male control, (3) pineal-fed female, and (4) female control rats of Litter 3. No appreciable difference in size can be noted between animals of the same sex.

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FIG. 1.

(Sisson and Finney: Effect of feeding pineal body on albino rat.)

SOURCE AND SIGNIFICANCE OF STREPTOCOCCI IN MARKET MILK.

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Considerable discussion has arisen as to the source and significance of streptococci in milk. Most of the observations were made before the use of the blood agar plate method or before the use of more accurate determinations of acid production became a common practice. New interest in the problem has been aroused since the outbreaks of septic sore throat attributed to contamination of the milk supply with streptococci pathogenic for man. A more thorough knowledge of milk streptococci, particularly those producing hemolysis in blood agar plates, is necessary. The source of such organisms is of particular interest.

Rogers and Dahlberg¹ undertook the study of milk streptococci in detail. They examined mixed milk, udder milk, feces, and saliva of cows. Blood agar plates were not used. The bouillon employed was prepared from beef extract to which dextrose, lactose, saccharose, mannite, raffinose, starch, and glycerol were added to make 1 per cent solutions. In all a large number of cultures were studied. The fecal streptococci were characterized by their ability to attack the sugars, including raffinose, and their inability to utilize alcohols. The salivary strains fermented dextrose, lactose, saccharose, mannite, and frequently raffinose. The amount of acid produced was lower than that obtained from the fecal streptococci. They divided the udder streptococci into two groups on the basis of the liquefaction of gelatin. One group, which they considered indistinguishable from *Streptococcus pyogenes*, fermented dextrose, lactose, and saccharose with an occasional culture breaking down mannite, starch, or inulin. In addition to liquefying gelatin, the other group attacked dextrose, lactose, saccharose, mannite, and generally fermented glycerol. From these studies the conclusion was reached that the streptococci found in market milk were of udder origin.

¹ Rogers, L. A., and Dahlberg, A. O., *J. Agric. Research*, 1913-14, i, 491.

Davis² in examining the market milk from nine dairies found hemolytic streptococci in eight samples; eight of the samples had been pasteurized. One of the pasteurized samples did not contain hemolytic streptococci. In the other seven they made up from 5 to 45 per cent of the flora. In one sample of certified (raw) milk 40 per cent of the bacteria were hemolytic streptococci. The data concerning fermentation are given for ten strains. They are compared with three strains of human origin and one culture isolated from a case of bovine mastitis. All the milk streptococci fermented dextrose, lactose, maltose, and salicin. Seven acidulated saccharose and one mannite. The titratable acidity in dextrose bouillon varied from 5.05 to 6.55 per cent. Two of the human cultures produced somewhat less acid than the milk strains (4.55 and 4.70 per cent), but the other produced more acid (6.25 per cent). The mastitis strain could not be differentiated by its cultural characters from the milk streptococci. However, it possessed considerable pathogenicity for rabbits. Davis points out that of the 85 strains studied none had properties which would justify his considering them of human origin. He concludes by stating that the milk strains are different from certain strains of hemolytic streptococci found at times in the diseased udders of cows, since the latter are virulent for rabbits and are from human sources.

The writer^{3,4} has shown that apparently normal cows may harbor in the udder, streptococci which are identical in all cultural characters and agglutination affinities with those causing mastitis. It was pointed out that in a herd in which a large number of cows suffered from mastitis many cows carried streptococci in the udder. From this observation it was assumed that many such streptococci would gain access to the milk supply.

To establish the possible types of streptococci which may appear in market milk, examinations of the vaginal discharges, saliva, feces, and skin of cows in a large herd were undertaken. The milk from this herd is of high quality and is sold in one large city as certified milk. In another city it is marketed as Grade A raw milk which is certified by a board of physicians. The usual precautions taken in the production of certified milk are maintained.

The Possible Sources of Streptococci.

In a previous paper⁴ the vaginal streptococci were considered in detail. At that time it was noted that hemolytic streptococci were not found on the normal vaginal mucosa. A considerable number

² Davis, D. J., *J. Infect. Dis.*, 1916, xix, 236.

³ Jones, F. S., *J. Exp. Med.*, 1918, xxviii, 253.

⁴ Jones, F. S., *J. Exp. Med.*, 1918, xxviii, 735.

of the non-hemolytic variety were isolated and studied. They fell into two principal groups, those attacking dextrose, lactose, saccharose, maltose, and mannite, and those fermenting the first four sugars but not fermenting mannite. Salicin fermentation predominated in each group.

Swabs were employed in isolating streptococci from the mouth and skin. The swabs were introduced into the mouth and rubbed over the tongue and mucous membranes. Within an hour they were agitated in 10 cc. of sterile 0.9 per cent sodium chloride solution. From this suspension three platinum loopfuls were inoculated into 12 cc. of melted 2 per cent agar prepared from veal infusion which had been cooled to 45°C. The mixture was plated with 1 cc. of defibrinated horse blood. The plate cultures were examined after incubation for 24 hours at 38°C.

For the skin examinations the lumbar region was chosen. Since the cows were confined in stanchions the possibility of contaminating this area with either feces or saliva was not considered great.

Feces were obtained as early after defecation as possible and suspended in the usual amount of salt solution and plated at once.

The cultures were inoculated into tubes containing 13 cc. of fermented veal infusion broth (+ 0.6 to +0.8 phenolphthalein) to which the test substances were added to make a 1 per cent solution. Titrations were made after 5 days incubation at 38°C.

In all, the saliva, skin, and feces of 45 cows were examined. Hemolytic streptococci have not been isolated from these regions. The saliva contains many streptococci. In all, thirty-seven strains have been isolated and studied. They comprise, however, an exceedingly heterogeneous group. Mannite fermentation is frequent and the proportion of raffinose and inulin fermenters is high. As a rule, low acid production in dextrose is characteristic. A tabulation of their characters has been omitted as it will be shown later that such streptococci have not been found in the milk.

From the skin thirteen cultures of streptococci have been isolated. Their cultural characters, given in Table I, are more uniform than those from the mouth. From Table I it will be noted that the characteristic streptococcus from the skin is a type which produces short chains in bouillon, acidulates milk, and ferments dextrose, lactose,

saccharose, maltose, raffinose, mannite, and salicin. Mannite is uniformly attacked to a lesser degree than the other substances. Inulin is not fermented. Gelatin is not liquefied.

TABLE I.

The Non-Hemolytic Streptococci from the Skin.

Culture No.	Grouping.*	Growth in bouillon.	Litmus milk.	Production of acid in.							
				Dextrose.	Lactose.	Saccharose.	Maltose.	Raffinose.	Inulin.	Mannite.	Salicin.
				per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Sk 2	Pairs; chains of 4.	Turbid.	Coagulated on boiling.	2.7†	2.4	2.8	2.7	2.6	0.2	0.9	2.8
" 11	Pairs and S. C.	"	" "	3.3	2.9	2.9	3.2	2.7	0.2	1.2	3.2
" 12	" "	"	" "	3.2	2.6	3.5	3.2	2.9	0.0	2.0	3.0
" 20	" "	"	Acid.	3.1	2.6	3.2	2.7	2.9	0.2	2.5	3.2
" 23	" "	"	Coagulated on boiling.	3.2	2.9	3.3	3.0	2.8	0.1	1.8	3.1
" 24	" "	"	" "	2.9	2.5	2.9	2.8	2.3	0.1	2.5	2.7
" 26	" "	"	" "	2.4	2.4	2.2	2.2	2.4	0.0	1.0	2.4
" 27	" "	"	" "	4.3	3.3	3.3	3.6	3.1	0.0	2.5	4.0
" 29	" "	"	" "	4.2	3.6	4.1	4.3	3.5	0.0	1.2	3.3
" 30	" "	"	" "	2.6	2.1	2.4	2.6	2.5	0.0	2.9	2.4
" 32	" "	"	" "	2.7	2.2	2.6	2.4	3.0	0.0	1.3	2.4
" 34	" "	"	" "	3.6	2.9	3.3	3.1	2.8	0.0	1.4	2.7
" 35	" "	"	" "	3.0	2.7	2.6	2.9	2.7	0.0	1.0	2.7

* The length of chains has been indicated as follows: S. C., 4 to 8 cocci; M. C., chains of 8 to 16; L. C., chains of more than 20.

† Only net acid production has been recorded in the tables. The addition of 0.7 to the figures in the various columns will give a close approximation of the total acidity.

Twenty-one strains of streptococci have been isolated from the feces. Their cultural characters are shown in Table II. The bulk of fecal streptococci fall into one large group. This group is characterized by the formation of large amounts of acid in dextrose, lactose, saccharose, maltose, raffinose, inulin, and salicin. Litmus milk is firmly coagulated and litmus is usually reduced. Gelatin is not liquefied. Two cultures, Nos. F 11 and F 20, belong to the skin group.

Since the vaginal, skin, and fecal streptococci possess specific characters rendering their classification definite, each type should be readily recognized if found in milk.

TABLE II.
The Non-Hemolytic Fecal Streptococci.

Culture No.	Grouping.	Growth in bouillon.	Litmus milk.	Production of acid in.							
				Dextrose.	Lactose.	Saccharose.	Maltose.	Raffinose.	Inulin.	Mannite.	Salicin.
				per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
F 4	Pairs and S. C.	Turbid.	Firmly coagulated.	7.3	5.1	6.5	6.3	6.3	7.3	0.0	5.5
" 6	" "	"	" "	8.3	4.9	7.1	7.6	7.0	6.5	0.0	5.0
" 18	" "	"	" "	7.2	5.4	5.6	7.0	5.8	7.0	0.0	5.0
" 21	" "	"	" "	6.4	4.6	5.7	5.7	5.7	6.6	0.0	5.5
" 28	" "	"	" "	6.4	5.0	5.5	5.9	5.7	6.6	0.1	5.0
" 29	" "	"	" "	5.3	4.4	5.2	4.9	5.1	6.0	0.0	4.5
" 30	" "	"	" "	5.7	4.2	5.3	5.7	5.5	5.7	0.0	5.3
" 31	" "	"	" "	6.5	6.4	5.8	5.8	5.1	6.4	0.0	6.0
" 32	" "	"	" "	6.2	4.0	6.0	5.6	6.0	6.7	0.0	5.2
" 33	" "	"	" "	7.2	4.5	5.9	5.8	5.7	6.4	0.0	5.4
" 34	" "	"	" "	5.9	4.6	5.4	5.8	5.8	6.0	0.0	5.4
" 35	" "	"	" "	5.9	4.3	5.8	5.9	6.5	6.5	0.0	5.6
" 36	" "	"	" "	6.4	4.7	5.7	6.1	4.9	6.3	0.0	5.1
" 37	" "	"	" "	6.0	4.2	6.0	5.2	5.4	6.0	0.0	4.6
" 39	" "	"	" "	6.4	4.5	5.3	4.5	6.5	6.0	0.0	5.3
" 40	" "	"	" "	6.4	4.0	5.5	5.3	6.1	6.3	0.0	5.1
" 2	" "	"	" "	7.4	5.8	5.9	6.2	3.3	0.0	0.0	5.8
" 11	" "	"	Coagulated on boiling.	3.2	2.4	3.1	3.1	2.9	0.1	1.8	3.0
" 20	" "	"	Acid.	3.9	3.0	2.7	3.1	3.0	0.1	1.4	3.5
" 16	M. C.	"	Unchanged.	4.1	0.0	4.1	4.3	3.2	2.8	3.4	4.0
" 46	Pairs and S. C.	"	Acid.	2.1	2.0	2.1	2.0	2.0	0.0	0.2	2.0

Examination of Market Milk for Streptococci.

Pint bottles of milk from the herd supply were chosen at random at various times. 1 cc. of milk was diluted in 9 cc. of sterile salt solution. After shaking vigorously, 0.5 cc. of the mixture was plated with 12 cc. of melted agar and 1 cc. of defibrinated horse blood. After incu-

bation for 24 hours at 38°C. the plates were examined. In all, twenty-six samples have been plated. The average for all the samples has been 2,850 organisms per cubic centimeter, of which 15.5 per cent have been streptococci. The proportion of streptococci varied over a wide latitude; the greatest number recorded was 45 per cent of the

TABLE III.
The Non-Hemolytic Streptococci from Milk.

Culture No.	Grouping.	Growth in bouillon.	Litmus milk.	Production of acid in.								Results of agglutination with mastitis streptococcus, serum dilution 1:1,000.
				Dextrose.	Lactose.	Saccharose.	Maltose.	Raffinose.	Inulin.	Mannite.	Salicin.	
				per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	
BM 8	L. C.	Clear.	Firmly coagulated.	3.4	2.8	2.8	3.0	0.0	0.1	0.1	2.3	+
" 13	"	"	" "	4.5	3.5	3.2	3.8	0.0	0.1	0.0	3.1	—
" 17	"	"	" "	4.5	3.4	3.3	3.0	0.0	0.1	0.0	3.2	(1:500 = ++)
" 27	M. C.	Turbid.	" "	4.3	3.6	3.3	3.4	0.0	0.1	0.0	3.2	+++
" 32	L. C.	"	" "	4.7	3.9	3.4	3.8	0.0	0.0	0.0	2.9	+++
" 39	"	"	" "	4.4	3.8	3.8	4.0	0.0	0.1	0.0	3.2	—
" 49	M. C.	"	" "	3.8	3.8	3.1	3.2	0.1	0.1	0.0	2.9	(1:500 = +)
" 53	L. C.	"	" "	4.4	3.9	3.9	4.0	0.0	0.0	0.0	3.8	+++
" 61	"	"	" "	4.5	3.6	3.5	4.1	0.1	0.1	0.1	3.8	+
" 69	S. C.	"	" "	4.6	3.7	4.4	4.2	0.0	0.1	0.0	3.7	—
" 72	M. C.	Clear.	" "	4.5	4.1	4.1	3.8	0.0	0.1	0.0	2.8	+
" 74	L. C.	"	" "	4.6	3.5	3.2	4.0	0.0	0.0	0.0	3.3	++
" 78	M. C.	Turbid.	" "	4.6	4.0	3.3	3.8	0.0	0.0	0.0	3.5	++
" 89	L. C.	"	" "	4.3	3.5	3.5	4.0	0.0	0.0	0.0	2.9	+++
" 92	M. C.	"	" "	4.3	3.5	3.3	3.5	0.0	0.0	0.0	3.3	+++
" 23	S. C.	"	Coagulated on boiling.	3.0	2.9	3.1	2.7	2.8	0.0	1.0	3.1	—

* +++ indicates complete agglutination; ++, marked clumping without entire clearing of the fluid; +, moderate agglutination. A negative reaction is noted as —.

total number of organisms. Of the 72 streptococci isolated, 56 are of the hemolytic type. The others are non-hemolytic. These figures indicate a greater proportion of hemolytic streptococci, although this is not necessarily the case. The hemolytic colonies are more

easily differentiated from the others and many of the deeper non-hemolytic colonies may have been overlooked. In Tables III and IV the cultural characters of the milk streptococci are given.

It will be observed that the non-hemolytic streptococci with one exception fall into the mastitis group. They all produce considerable acid in dextrose, lactose, saccharose, maltose, and salicin. Milk is firmly coagulated but litmus is not reduced. In addition, all but one agglutinate with their specific group serum. This serum was obtained by the immunization of a cow with a strain of non-hemolytic mastitis streptococcus. Strain BM 23 agrees in neither its cultural nor immunological characters with the other organisms; it is probably from the skin, since it ferments dextrose, lactose, saccharose, maltose, raffinose, mannite, and salicin.

Of 56 strains of hemolytic streptococci, 43 agree in their cultural characters and agglutination affinities with the hemolytic types most frequently associated with bovine mastitis. Like the non-hemolytic group, they produce considerable acid in dextrose, lactose, saccharose, and maltose. Salicin may or may not be fermented. Milk is firmly coagulated without the reduction of litmus. These high acid-producing strains (both hemolytic and non-hemolytic) occurred in the milk in large numbers. They make up the bulk of the streptococcic flora.

The cultural characters of the other thirteen strains differ from those associated with mastitis. They ferment dextrose, lactose, saccharose, and maltose, but none has attacked inulin, raffinose, mannite, or salicin. One culture fails to attack saccharose. Milk is coagulated but not firmly. The curd is flocculent. Litmus is often partially reduced. The amount of acid produced in dextrose is considerably lower than that recorded for the mastitis type. In addition, the hydrogen ion concentration is higher, pH 5.0 to 5.3. Gelatin and coagulated serum are not liquefied. An additional point of differentiation between members of this group and those of the mastitis group is the difficulty with which they are suspended in 1 per cent sodium chloride solution. The mastitis strains all readily enter into suspension. The others sediment spontaneously within 3 or 4 hours. When freshly isolated many of these strains grow with difficulty in fermented or plain bouillon, even when carbohydrate is added; the addition of 5 to 10 per cent of sterile horse serum to the media insures

TABLE IV.
The Hemolytic Streptococci from Milk.

Culture No.	Grouping.	Growth in bouillon.	Litmus milk.	Production of acid in.								Results of agglutination test with mastitis streptococcus, serum dilution 1:200.	Hydrogen ion concentration in distilled water, after 24 hours fermented broth.
				Dextrose.	Lactose.	Saccharose.	Maltose.	Raffinose.	Inulin.	Mannite.	Saltin.		
				per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent		pH
BM 1	M. C.	Turbid.	Firmly coagulated.	4.7	3.8	3.9	4.3	0.0	0.0	0.0	0.1	++	4.4
" 5	"	"	"	4.7	3.8	4.0	4.3	0.1	0.1	0.1	0.1	++	4.5
" 6	"	"	"	4.5	3.9	3.8	4.0	0.1	0.1	0.0	0.0	++	4.7
" 9	S. C.	"	"	4.2	3.8	3.3	3.5	0.1	0.0	0.0	0.0	++	4.7
" 10	M. C.	"	"	3.9	3.6	3.9	4.0	0.0	0.0	0.0	0.0	++	4.5
" 11	"	"	"	4.6	3.9	3.5	4.0	0.1	0.1	0.0	0.0	++	
" 14	"	"	"	5.0	4.1	4.0	4.1	0.1	0.0	0.0	0.0	++	
" 15	L. C.	"	"	4.7	4.0	4.1	3.6	0.0	0.1	0.0	0.0	++	
" 16	"	"	"	4.4	3.8	4.0	4.2	0.1	0.1	0.0	0.0	++	
" 18	"	"	"	4.8	3.7	3.9	4.2	0.1	0.0	0.0	0.1	++	
" 19	"	"	"	4.0	3.7	3.8	3.9	0.2	0.2	0.1	0.1	++	
" 20	M. C.	"	"	4.6	3.7	3.9	4.0	0.0	0.1	0.0	0.1	+	4.5
" 21	L. C.	"	"	4.9	3.8	3.9	4.3	0.0	0.0	0.1	0.1	++	
" 25	"	"	"	4.1	3.2	3.5	3.5	0.0	0.1	0.0	0.0	++	
" 33	M. C.	Clear.	"	4.0	3.8	3.6	3.6	0.0	0.1	0.0	0.0	++	
" 35	"	Turbid.	"	4.9	4.0	3.9	4.2	0.0	0.0	0.0	0.1	++	
" 37	L. C.	"	"	5.4	3.8	3.8	4.6	0.0	0.0	0.0	0.0	++	4.6
" 40	S. C.	"	"	4.6	3.9	4.0	4.3	0.1	0.0	0.1	0.0	++	
" 42	M. C.	"	"	4.5	4.0	3.9	4.0	0.0	0.0	0.0	0.0	++	
" 43	"	"	"	5.3	4.4	4.4	4.8	0.0	0.0	0.1	0.0	+	4.4
" 45	"	"	"	4.9	4.0	4.0	4.0	0.0	0.0	0.0	0.0	++	
" 46	S. C.	"	"	4.8	4.0	4.0	4.1	0.0	0.0	0.0	0.0	++	
" 47	L. C.	"	"	4.5	3.9	3.9	4.3	0.0	0.0	0.0	0.0	++	

BM 51	M. C.	Turbid.	Firmly coagulated.	4.7	3.8	3.9	4.5	0.0	0.0	0.0	0.0	0.0	0.0	++		
" 52	"	"	"	4.7	3.9	4.1	4.4	0.0	0.0	0.1	0.0	0.1	0.0	++		
" 54	L. C.	"	"	4.6	4.1	4.1	4.5	0.0	0.1	0.0	0.1	0.0	0.1	++		
" 56	"	"	"	4.7	4.1	3.8	4.1	0.0	0.0	0.0	0.0	0.1	0.0	++		
" 57	S. C.	"	"	4.0	3.8	3.8	4.1	0.0	0.1	0.0	0.0	0.0	0.0	++		
" 59	L. C.	"	"	4.5	4.0	3.8	4.1	0.0	0.0	0.0	0.0	0.0	0.0	++		
" 62	M. C.	"	"	4.6	4.1	4.1	4.5	0.1	0.0	0.1	0.1	0.1	0.1	++		
" 66	L. C.	"	"	4.5	4.1	4.1	4.2	0.0	0.1	0.1	0.0	0.1	0.0	++		
" 70	M. C.	"	"	4.8	4.0	4.1	4.4	0.1	0.0	0.0	0.0	0.0	0.0	++		
" 73	"	"	"	4.3	3.4	4.0	3.8	0.0	0.0	0.1	0.1	0.1	0.1	++		
" 76	S. C.	"	"	4.6	4.1	4.1	4.1	0.0	0.0	0.0	0.0	0.0	0.0	++		
" 81	M. C.	"	"	4.9	4.0	3.8	5.0	0.0	0.0	0.0	0.0	0.0	0.0	++		
" 85	"	"	"	5.0	3.9	3.8	4.4	0.0	0.0	0.0	0.0	0.0	0.0	++		
" 87	L. C.	Clear.	"	5.0	3.7	3.7	4.0	0.0	0.0	0.0	0.0	0.0	0.0	++		4.7
" 90	"	"	"	4.8	4.3	4.0	3.9	0.0	0.1	0.0	0.0	0.0	0.0	++		4.5
" 2	"	"	"	4.2	3.9	4.0	3.8	0.1	0.0	0.1	0.1	2.5	2.5	++		
" 4	"	"	"	4.4	3.9	3.9	4.1	0.1	0.0	0.1	3.1	3.1	3.1	++		
" 24	"	Turbid.	"	4.7	3.9	4.3	4.0	0.1	0.0	0.0	2.4	2.4	2.4	++		
" 29	"	"	"	4.9	4.6	4.1	4.2	0.0	0.0	0.0	3.2	3.2	3.2	++		
" 63	"	"	"	4.4	4.3	4.5	4.0	0.1	0.0	0.0	2.5	2.5	2.5	++		
" 17A	"	Clear.	Coagulated on boiling.	3.4	3.0	2.6	3.3	0.0	0.0	0.0	0.0	0.0	0.0	++	Spontaneous precipitation.	5.2
" 20	S. C.	Turbid.	Firmly coagulated.	3.3	3.0	2.5	3.3	0.1	0.0	0.0	0.0	0.0	0.0	++	"	5.1
" 30	M. C.	Clear.	Coagulated, not firmly.	3.6	2.7	1.1	3.5	0.0	0.0	0.0	0.0	0.0	0.0	++	"	5.2
" 44	"	Turbid.	"	3.3	2.7	2.6	3.3	0.0	0.1	0.0	0.0	0.0	0.0	++	—	5.2
" 58	S. C.	"	"	3.4	3.4	2.7	2.8	0.0	0.1	0.0	0.0	0.0	0.0	++	Spontaneous precipitation.	5.2
" 60	"	"	"	3.4	3.0	1.8	3.1	0.0	0.1	0.0	0.0	0.0	0.0	++	"	5.0
" 64	L. C.	Clear.	"	3.1	3.0	2.4	3.0	0.0	0.0	0.0	0.0	0.0	0.0	++	"	5.2
" 67	"	"	"	3.3	3.1	1.2	3.0	0.0	0.0	0.0	0.0	0.0	0.0	++	"	5.3
" 77	M. C.	Turbid.	"	3.5	2.9	2.9	3.7	0.0	0.0	0.0	0.0	0.0	0.0	++	"	5.2
" 82	S. C.	"	"	3.5	3.1	2.8	3.6	0.0	0.0	0.0	0.0	0.0	0.0	++	"	5.2
" 84	"	"	"	3.6	2.9	2.8	3.6	0.0	0.0	0.0	0.0	0.0	0.0	++	"	5.0
" 88	M. C.	"	"	3.0	2.9	2.6	3.5	0.0	0.0	0.1	0.0	0.1	0.0	++	"	5.2
" 71	"	"	"	3.3	3.1	0.0	3.3	0.0	0.0	0.0	0.1	0.0	0.1	++	"	5.2

moderate growth. Growth, however, takes place slowly. In a number of instances several attempts were made before fermentation was obtained in saccharose. The colonies in blood agar plates are smaller than those produced by the members of the larger group. The surface colonies rarely reach 1 mm. in diameter. They are sharply raised and surrounded by a clear zone of hemolysis. The deep colonies are exceedingly small, biconvex or ovoid in shape, and produce clear hemolytic areas 1.5 to 4 mm. in diameter after 24 hours incubation. After 48 hours the diameter of the hemolytic area is frequently doubled or trebled.

Considerable differences in the morphology have been observed. Chains of 6 to 15 elements are usually observed in liquid media. The individual elements are usually round, slightly elongated, and even rod-shaped. In the water of condensation of blood agar cultures the organisms appear as diplococci and short rods. Clubbed ends are frequently observed. All forms retain the stain by Gram's method. The morphology of strains grown on artificial media for some time becomes more fixed, since the organisms grow in bouillon as typical short chained streptococci.

Five of the cultures from this group chosen at random were tested for pathogenicity in rabbits. 1 cc. of a 24 hour serum bouillon culture was injected into the ear veins. The animals were not appreciably affected. Their temperatures ranged well within the normal limits. Two of the rabbits were killed 15 days after injection. Necropsy failed to reveal localization either in the heart valves, the joints, or the viscera. Eight other cultures in 0.1 cc. doses were injected into the peritoneal cavities of white mice. The mice remained well.

Members of this group appeared in half the samples of milk examined, but in very small numbers; usually only one, two, or three colonies developed in plate cultures.

The low acid-producing strains in market milk had not been found in any of the regions examined. In previous studies such organisms had not been noted in plates made directly from udder milk. It was determined to trace their source back from the bottled milk. On this particular dairy farm it is customary to collect the milk from individual cows into 40 quart cans. These cans are removed from the barns, and sent to the creamery as soon as they are filled. The con-

tents of the cans, on the average, are made up of the mixed milk of five or six cows. Samples were obtained from the various cans from each barn. Characteristic colonies of the low acid-producing streptococci were observed in certain samples of can milk. This observation pointed to one of three sources of entrance: udder infection, exfoliations from the skin of the udder, or contamination by the milker during milking. Accidental contamination from exfoliations from the skin or from the milker did not seem probable, especially as covered milk pails are used. The skin of the udder of 50 cows was examined with negative results. It was determined to examine the udder milk from cows whose mixed milk was known to contain streptococci of the type sought. The search proved more difficult than at first supposed. Many udders were found infected with the typical bovine types, but comparatively few harbored the low acid producers. Of 50 cows in one barn the milk from two revealed organisms of this type. Usually, however, one finds at least one in 50 harboring the low acid-producing streptococci. The number of colonies which develop from 1 cc. of milk is usually small (40 to 80). In one instance, however, plates from the milk revealed 900 colonies per cubic centimeter. Undoubtedly more udders are invaded than indicated, since these streptococci are exceedingly delicate and are crowded out in the plates by the ordinary udder streptococci and other organisms. Higher dilutions in milk with high counts entirely eliminate them from the plate cultures since they occur only in small numbers.

Another streptococcus similar in many respects to the low acid-producing strains was found in milk drawn directly from the udder. It, however, ferments mannite and salicin in addition to dextrose, lactose, saccharose, and maltose. Such strains are also low acid producers and grow poorly in plain or fermented bouillon. The colonies and hemolytic characters in blood agar plates are indistinguishable from those noted for the low acid-producing streptococci.

DISCUSSION.

The predominating types of streptococci in the market milk are those of mastitis. It is true that fecal and skin streptococci may gain access to the milk, but they are practically absent in fresh milk

if it is kept under proper conditions. It is conceded, however, that certain of these streptococci may play an important part in the souring of milk; as for instance, the high acid-producing fecal strains.

Heinemann⁵ in considering the significance of streptococci in milk came to the conclusion that *Streptococcus lacticus*, supposedly the most common type of milk streptococci, agrees in its morphological and cultural characters with pathogenic, fecal, and sewage streptococci. Miller⁶ reached the same conclusion. He asserted that *Streptococcus pyogenes* and *Streptococcus lacticus* are indistinguishable by present methods of study. Rogers and Dahlberg reached the same conclusion in regard to certain udder streptococci.

The most frequent types of streptococci which I have observed are identical with those causing mastitis. It has been shown repeatedly that such streptococci are found in the udder before clinical manifestations of the disease appear and may persist for long periods after symptoms have subsided. That such streptococci have been isolated from market milk is shown by Davis' protocols. The only point of difference between his milk streptococci and a strain from a case of mastitis was in the virulence of the latter for rabbits. The writer has never isolated strains of mastitis streptococci of purely bovine origin which possessed high pathogenic properties for rabbits. The question is raised whether or not many belonging to the so called *Streptococcus lacticus* group may not be identical with mastitis streptococci.

The experiments of Mathers⁷ have a distinct bearing on this point. He injected human, mastitis, and milk streptococci into the udders of cows. The human and mastitis strains produced a severe mastitis which became chronic. The non-hemolytic *Streptococcus lacticus* gave rise to an acute mastitis which displayed all the symptoms observed in spontaneous mammitis (swelling, heat, pain, and purulent milk). The injection of a culture of a hemolytic streptococcus from milk gave much the same result. The inflammation resulting from these injections disappeared within 2 weeks. The fact remains, however, that both supposedly non-pathogenic cultures produced disease. It is admitted that the inflammation is of short duration, but such is not infrequent in certain cases of spontaneous infection.

⁵ Heinemann, P. G., *J. Infect. Dis.*, 1906, iii, 173.

⁶ Miller, W. W., *Bull. Hyg. Lab., U. S. P. H.*, No. 41, 1908, 479.

⁷ Mathers, G., *J. Infect. Dis.*, 1916, xix, 222.

It is difficult to harmonize the results obtained by Savage⁸ in England with those obtained by me. The method of handling and caring for cows, the precautions taken to avoid contamination of the milk, etc., make such comparisons impossible. The types of streptococci producing mastitis in the district in which Savage's observations were made differ to a considerable degree from those which I have isolated. However, he brings out the point that most of the streptococci which he found in milk are of udder origin, and considers that the udder group is intermediate between the fecal and mastitis streptococci.

The group of low acid-producing hemolytic streptococci is interesting. Their hydrogen ion concentration readings are significant. According to Avery and Cullen's⁹ observations, they fall well within the human group. In fact, in Table VIII of their paper the readings of three strains (M. 53, M. 86, and J. 1), isolated by the writer, are given. These strains were obtained from milk. At that time it was stated that they were not associated with mastitis, but might have been human contaminations, or arisen from the skin or feces of the cow. The latter postulation has been disproved, since hemolytic streptococci have not been found on the skin or in the feces of the cows supplying milk in this herd. These strains produce about the same amount of titratable acid in dextrose as the human streptococci. They differ from pathogenic human streptococci in character of the colonies produced on blood agar and in their ability to coagulate milk. They possess no virulence for mice and rabbits. It is possible, however, that such streptococci may be of human origin. Holman¹⁰ designates streptococci which are hemolytic and ferment lactose but do not attack mannite, inulin, or salicin as *Streptococcus anginosus* (Andrewes and Horder). Several have reported the presence of such organisms in the human nose, throat, and tonsils. Perhaps these organisms gain access to the udder from the milker. At any rate they do not possess severe invasive characters for cattle, since their numbers are held in check in the udder. They differ from the non-

⁸ Savage, W. G., *Rep. Med. Off., 1906-07, Local Gov. Bd., Appendix B, No. 4*, 1908, 3.

⁹ Avery, O. T., and Cullen, G. E., *J. Exp. Med.*, 1919, xxix, 215.

¹⁰ Holman, W. L., *J. Med. Research*, 1916, xxxiv, 377.

salicin-fermenting mastitis streptococci both in their hemolytic properties and in their inability to produce as much acid.

On morphological grounds they resemble diphtheroids, but have been placed with the streptococci for several reasons. In addition to forming chains in bouillon, they produce the characteristic hemolytic areas associated with hemolytic streptococci. Their fermentative characters resemble those of streptococci. In cultures that have grown on artificial media for considerable periods they produce in bouillon only chains of cocci.

Both the mastitis and the other group of streptococci have been observed in the market milk from this farm during the past 2 years. During this time diseases traceable to this milk supply have not been reported. This evidence points to the low pathogenicity of either group for consumers of milk. Either species may be eliminated as a source of severe epidemics of milk-borne sore throat.

SUMMARY.

The principal source of streptococci in milk is the cow's udder. The udder streptococci fall into two broad groups; those of the larger group agree in cultural characters and agglutination affinities with mastitis streptococci; the smaller group is composed of low acid-producing streptococci. The streptococci of the latter group produce clear zones of hemolysis about surface and deep colonies in horse blood agar plates. They attack dextrose, lactose, saccharose, and maltose, but do not ferment raffinose, inulin, mannite, or salicin. Acid production in dextrose by the members of this group is about the same as that produced by human streptococci under the same conditions. The limiting hydrogen ion concentration for these pleomorphic udder streptococci in dextrose serum bouillon is within the limits of the limiting hydrogen ion concentration observed by Avery and Cullen for human streptococci.

All the streptococci from the vagina, saliva, skin, and feces have been non-hemolytic. Those from the saliva form a heterogeneous aggregation in which individuals fermenting raffinose, inulin, and mannite predominate. From the skin a characteristic streptococcus has been found. It produces acid in dextrose, lactose, saccharose, mal-

tose, raffinose, mannite, and salicin, but fails to acidulate media containing inulin. The fecal streptococci are characterized by the formation of large amounts of acid in dextrose, lactose, saccharose, maltose, raffinose, inulin, and salicin. Mannite is not fermented. Neither the fecal nor the skin streptococci have been isolated from the bottled milk with any great frequency.

EXPERIMENTAL STUDIES ON DIABETES.

SERIES I. PRODUCTION AND CONTROL OF DIABETES IN THE DOG.

1. GROSS ANATOMIC RELATIONS OF THE PANCREAS AND DIABETES.

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The basis of the present studies has been a form of diabetes produced by removal of the greater part of the pancreas of animals, leaving a remnant about the duct secreting normally into the duodenum, thus avoiding the rapidly fatal cachexia of total pancreatectomy and also the pancreatic sclerosis and deficient digestion of Sandmeyer diabetes and affording a very close and satisfactory reproduction of the clinical disorder.¹ Because of the importance of relations between pancreas mass and body mass, and between the total pancreas and the size of remnant with which diabetes occurs, a summary will first be given of these anatomic facts in dogs, taken from the records of all the experiments suitable for this purpose.

Accuracy of Estimation of the Size of the Pancreas Remnant.

The method of operation and estimation of the remnant has been previously described.² In brief, a piece of the removed tissue is trimmed to imitate the remnant left *in situ*; this piece and the total tissue removed are weighed, and the absolute and relative weights of the remnant and total pancreas are thus reckoned. It is important to combine sight and touch to obtain the greatest accuracy, for feeling the comparative thickness and mass often corrects mistakes of the eye. Though practice is important, the range of error was practically constant throughout this series, and differences depended

¹ All operations were performed under ether anesthesia.

² Allen, F. M., Studies concerning glycosuria and diabetes, Cambridge, 1913, Chapter X.

not so much upon care, skill, or chance as upon differences in the shape of the pancreas. When, as frequently, a piece can be cut from directly beside the remnant, of practically the same shape and consistency, the agreement is generally close; but when a piece of different form and structure must be used, the error is likely to be greater.

As shown in Table I, 68 dogs freshly dead from natural or experimental causes were subjected to the usual operation and the actual remnant was immediately removed and compared with the estimated one. The total of the pancreas weights of the 68 dogs was 1,812.8 gm. The sum total of the estimated remnants was 197.5 gm., of the actual

TABLE I.
Accuracy of Estimation of the Size of the Pancreas Remnant.

Material.	No. of animals.	Combined weight of.			Maximum individual error		
		Total pancreas.	Estimated remnants.	Actual remnants.	Absolute.	Relative, as fractions of total pancreas.	
		gm.	gm.	gm.		Estimated remnant.	Actual remnant.
Dead dogs.....	68	1,812.8	197.5	196.8	1.1	$\frac{1}{4}$	$\frac{1}{11} - \frac{1}{12}$
“ puppies.....	12	74.0	9.1	9.2	0.4	$\frac{1}{9} - \frac{1}{10}$	$\frac{1}{7} - \frac{1}{8}$
Total pancreatectomies.....	18	419.7	45.2	43.4	0.8	$\frac{1}{8}$	$\frac{1}{10}$

remnants 196.8 gm. The greatest individual error occurred when the total pancreas weighed 70.7 gm., the estimated remnant 5.1 gm., and the actual remnant 6.2 gm. Thus, the absolute difference between the remnants was 1.1 gm., and when expressed as usual in the form of fractions, the estimated remnant was approximately $\frac{1}{4}$ and the actual remnant between $\frac{1}{11}$ and $\frac{1}{12}$ of the total pancreas. The absolute error is naturally apt to be greatest when, as here, the dog, pancreas, and remnants are large; but only rarely is the relative error sufficient to change the denominator of the fraction by more than one digit. In this series of 68 dogs, the estimated and actual remnants were equal or within 0.1 gm. of each other in 25 instances. In the other 43 animals, the estimated remnant weighed more than

the actual remnant in 22 cases, and less in 21. The difference between the estimated and actual remnants was 0.2 gm. in 17 instances, 0.3 gm. in 9 instances, 0.4 gm. in 6 instances, 0.5 gm. in 4 instances, 0.6 gm. in 2 instances, 0.7 gm. in 1 instance, 0.8 gm. in 1 instance, 0.9 gm. in 2 instances, and 1.1 gm. in the extreme instance mentioned.

A similar procedure was carried out upon twelve dead puppies, ranging in age from 2 weeks to 10 months. As the animals and organs were smaller, the absolute error was less, but the relative error was about the same. It was greatest in the largest pup, which was 10 months old, and in which the pancreas weighed 13.8 gm., the estimated remnant 1.4 gm., and the actual remnant 1.8 gm. In every other instance the agreement was within 0.1 gm.

The conditions of operation upon living animals were fully reproduced when partial pancreatectomy was performed in the course of total pancreatectomy upon eighteen dogs. The general summary is shown in Table I. The estimated and actual remnants were equal or within 0.1 gm. of each other in 4 instances. They differed by 0.2 gm. in 3 instances, by 0.3 gm. in 3 instances, by 0.4 gm. in 2 instances, by 0.6 gm. in 5 instances, and by 0.8 gm. in 1 instance. This maximum error occurred when the total pancreas weighed 31.4 gm., the estimated remnant 3.9 gm., $= \frac{1}{8}$, and the actual remnant 3.1 gm., $= \frac{1}{10}$ of the total pancreas.

It may be concluded from these figures that the method of estimation is exact enough to be used for statistical studies. But close as is the agreement on the average and in the majority of cases, there is a large minority of errors of a magnitude sufficient to determine the occurrence or absence of diabetes. Accordingly, if the influence of any agencies in causing or preventing diabetes is to be tested with the aid of this operative method, these possible limits of error must be considered in judging the results.

Body Weight, Pancreas Weight, and the Tendency to Diabetes.

If the operative estimates are accepted as accurate to the degree above shown, a series of dogs subjected to partial pancreatectomy is available for analysis (Table II).

TABLE II.
Analysis of Results of Partial Pancreatectomies.

No. of dogs.	Body weight.	Pancreas weight.			Weight of pancreas per kilo of body weight.			Maximum fraction of pancreas with which diabetes occurred.
		Average.	Maximum.	Minimum.	Average.	Maximum.	Minimum.	
	kg.	gm.	gm.	gm.	gm.	gm.	gm.	
1	2	9.0			4.50			
2	3	9.9	10.0	9.7	3.30	3.33	3.23	
8	4	12.1	16.8	8.9	3.02	4.20	2.22	$\frac{1}{2} - \frac{1}{8}$
18	5	14.2	20.5	8.7	2.84	4.10	1.74	$\frac{1}{2}$
20	6	15.5	18.2	11.2	2.59	3.03	1.87	$\frac{1}{2}$
23	7	17.5	29.4	10.9	2.50	4.20	1.56	$\frac{1}{2}$
26	8	19.7	28.3	12.6	2.46	3.54	1.58	$\frac{1}{2} - \frac{1}{4}$
33	9	22.0	31.1	16.5	2.44	3.45	1.83	$\frac{1}{10}$
35	10	22.3	31.3	14.0	2.23	3.13	1.40	$\frac{1}{2}$
25	11	25.5	38.9	17.3	2.32	3.54	1.57	$\frac{1}{2} - \frac{1}{5}$
36	12	25.7	42.5	15.5	2.14	3.54	1.30	$\frac{1}{2}$
27	13	27.8	39.4	18.7	2.14	3.03	1.44	$\frac{1}{2}$
23	14	30.2	44.1	20.5	2.16	3.15	1.46	$\frac{1}{2}$
34	15	31.4	50.3	20.6	2.09	3.35	1.37	$\frac{1}{2}$
22	16	35.9	63.4	27.3	2.24	3.96	1.71	$\frac{1}{2}$
13	17	33.7	50.3	26.6	1.98	2.96	1.56	$\frac{1}{2} - \frac{1}{5}$
27	18	36.5	49.6	23.1	2.03	2.76	1.28	$\frac{1}{2}$
13	19	34.8	52.9	28.1	1.83	2.78	1.48	$\frac{1}{2}$
11	20	35.5	46.1	23.5	1.77	2.30	1.17	$\frac{1}{2}$
7	21	41.5	54.5	29.1	1.98	2.60	1.39	
2	22	35.1	38.5	31.8	1.59	1.75	1.45	$\frac{1}{2} - \frac{1}{4}$
6	23	46.5	54.7	38.3	2.02	2.38	1.66	$\frac{1}{2}$
5	24	45.0	51.9	39.4	1.87	2.16	1.64	$\frac{1}{2}$
2	25	66.3	94.4	38.0	2.65	3.77	1.52	
7	26	54.2	67.5	43.5	2.08	2.60	1.67	$\frac{1}{2} - \frac{1}{10}$
1	27	51.1			1.89			
1	28	40.8			1.46			
2	29	73.8	95.3	52.0	2.54	3.28	1.79	
1	30	35.9			1.19			
1	31	70.7			2.28			
2	34	72.8	88.9	56.8	2.14	2.62	1.67	
1	36	58.9			1.63			$\frac{1}{6}$
1	37	82.8			2.24			
1	42	81.8			1.95			

The weight of the pancreas ranged from a minimum of 1.17 gm. to a maximum of 4.5 gm. per kilo of body weight. These figures are derived from animals which were chosen as apparently normal in all

respects, free from any disturbing factors such as notable obesity or emaciation, extreme age or youth, pancreatic or other disease, etc., and in which, moreover, the normality of the pancreatic tissue was in a high proportion of instances confirmed by microscopic examination. They are therefore of statistical value from the pure anatomic standpoint, and also as a basis of comparison for some of the ensuing studies.

A detailed record was kept of the sex, apparent age, breed, color and character of coat, and other characteristics of all animals. Analysis indicates (*a*) that sex is immaterial, and also (*b*) that in adult animals variations of age are without perceptible influence in regard to the pancreas weight. (*c*) No breeds were found with characteristically large or small pancreas. (*d*) On the other hand, the numerous crosses and admixtures of different breeds were suggestive. Anyone handling large numbers of dogs will observe that while characters are generally blended, the size and form of organs are among those which are sometimes transmitted almost like Mendelian units. Thus, the large square bulldog head with its undershot jaw may be found on a fox-terrier body, and examples of obvious bastardy in legs, tail, coat, and other features are too common to mention. The uncertain ancestry of all the animals and the mixture in the majority make tabulation or exact conclusions impossible for both (*c*) and (*d*). But there is no doubt that the variations in size due to cross-breeding pertain also to visceral organs. One of the clearest examples is the occasional finding of the deep thorax and large lungs of a greyhound in a mongrel with the other characters of some other breed or mixture. The impression was gained that in the crossing of large and small dogs, the size of the pancreas in the offspring is usually a blend roughly proportioned to the body weight, but in some instances it is chiefly derived from either the large or the small parent, so that the ratio of pancreas weight to body weight is altered, and the animal has an exceptionally large or small pancreas in proportion to the size of its body.

Dogs offer unique advantages for tracing a range of relations between body weight and pancreas weight. No other mammal exhibits such a gamut of sizes as here represented between the 2 and 42 kilo weights for normal adults of the same species. In a broad and

general way, with allowance for the exceptions mentioned in the preceding paragraph, the table indicates a lowering of the proportional size of the pancreas as the body weight rises. The rule in this general sense applies to all three columns, of average, maximum, and minimum weights. In other words, small dogs generally possess more pancreas tissue in proportion to their body mass than large dogs. According to well known laws, the basal metabolism of small dogs per kilo is higher than that of large dogs, and with allowances for activity, warmth of coat, and other modifying factors, their actual daily metabolism in proportion to weight is doubtless higher. As one of the principal studies of the entire investigation pertained to the relation of the pancreatic function respectively to the body mass and to the metabolism, it was interesting to observe that dogs seem normally to be provided with pancreatic tissue rather in proportion to their metabolism than to their body mass.

The observations were also directed to determine whether a uniform mathematical law can be established for the occurrence of diabetes whenever a fixed proportion of the pancreas is removed, or whether general or individual exceptions exist. With regard to the statements in the preceding paragraph, it might be inquired whether small dogs have a larger endowment of pancreas corresponding to larger needs, or merely a greater margin of safety. It is known from veterinary literature that dogs are occasionally subject to spontaneous diabetes. In human pathology, a tendency to diabetes has sometimes been attributed either to small size of the pancreas in gross, or to deficiency of islands. In view of the impracticability of any extensive counting of islands, there was a question whether dogs having a small pancreas would show a corresponding susceptibility to diabetes, or whether differences in the functional capacity of equal masses of pancreatic tissue would be demonstrable, due to variations in island content or other differences. Dogs exhibit contrasts of nervous and phlegmatic temperaments almost as extreme as those of men. Also some varieties of dogs have resulted from close inbreeding, and special characters have sometimes been cultivated at the price of constitutional vigor. As shown in Table II, wide differences were observed in the size of the pancreas remnant with which diabetes occurred; but these were rare and did

not conform to any of the causes above considered. Special attention was paid to the few instances in which diabetes occurred with $\frac{1}{6}$ to $\frac{1}{4}$ of the pancreas present, but only after several years was it demonstrated that the explanation lay in inflammatory changes, as described in a paper to be published later. For the most part, large dogs develop diabetes more readily and are more satisfactory for the purpose than small ones, but accessory factors enter in. The small pancreas remnant in a small dog is traumatized in operation to a relatively greater extent than the larger remnant of a large dog. This difference is overbalanced by the greater vigor and resistance of large dogs. The small ones are more subject to loss of appetite, cachexia, and distemper, which interfere with the development of active diabetes. Owing to the modifying influences, no uniform rule can be deduced from the last column of Table II. The present figures, covering a larger number of animals, confirm previous findings² that, with few exceptions, mild diabetes occurs when the remnant is $\frac{1}{8}$ to $\frac{1}{9}$ of the pancreas, and severe diabetes when the remnant is about $\frac{1}{10}$ of the pancreas. The internal secretory capacity of equivalent fractions of pancreatic tissue is uniform as far as these observations could determine, with no sign of variations due to gross size of the organs, differing island content, nervous control, or constitutional vigor or degeneracy. Except for inflammatory changes as mentioned, no special tendency to diabetes was evident in any dog of the series.

The observations outline the limits of error under this procedure. A demonstration of the influence of any agency in producing or preventing diabetes, by a comparison of test animals and controls, must rest upon uniform results in a considerable series, and the differences in question must generally amount to several grams of pancreas tissue. Other experiments have shown that a partially depancreatized animal, which has merely a lowered tolerance and cannot be made diabetic by any quantity or duration of feeding, may be so close to the verge that diabetes results from the removal of only a fraction of a gram of additional pancreatic tissue. Also definite variations in the severity of diabetes may be produced, for

example by overfeeding. The most delicate method, therefore, consists in tests upon the same rather than upon different animals. Due attention must always be paid to accidental modifying factors, especially body weight, pancreatitis, and cachexia.

Hypertrophy of the Pancreas Remnant.

The former series of experiments contained several examples of great hypertrophy of the pancreas remnant. Thus, in Dog 104³ the pancreatic tissue left at the original operation on Oct. 1 was estimated at less than 6 gm. On Nov. 27, 6 gm. of tissue were removed, and the remnant found at autopsy on Dec. 23 weighed 8.1 gm. Also⁴ in Dog 151 the remnant estimated at operation on Nov. 21 was 3.2 gm., while that found at autopsy on Dec. 4 was 11.3 gm.; and in Dog 152 the remnant estimated at operation on Nov. 24 was 4.4 gm., while that found at operation on Dec. 5 was 10.4 gm. In Dog 125⁵ the remnant estimated at operation on Oct. 26 was 2.4 gm., and that found at autopsy on Nov. 8 was 7.7 gm. In Dog 148⁶ the remnant estimated at operation on Nov. 16 was 3.3 gm., and that found at autopsy on Dec. 19 was 13.3 gm.

Hypertrophy to such an extreme degree is highly exceptional. Tables were compiled from a series of dogs which had undergone removal of most of the pancreas in single operations and were free from any known disturbing influence. The data concerning alterations in weight of the pancreas remnant are summarized in Table III.

If ± 0.4 gm. is accepted as a rough estimate of the possible limits of error, it is seen that in 35 instances the weight of the remnant remained practically stationary. The 17 cases of atrophy represented essentially traumatic fibrosis. Hypertrophy occurred in the majority of cases in the total series, but was generally slight, so that in only 12 instances was the remnant found more than doubled in size. To save reproduction of extensive detailed data, the following points may be noted as developed by analysis.

Occurrence.—Changes occur in the size of the pancreas remnant beyond any possible error of estimation. In calculations of the proportion of pancreas removed, the operative estimate should be used in preference to the weight of tissue at autopsy.

³ Allen,² pp. 485–486.

⁴ Allen,² p. 490.

⁵ Allen,² p. 491.

⁶ Allen,² p. 959.

State of Nutrition.—Precautions were taken to make sure that the changes in weight did not represent mere differences in fullness and emptiness of the acini. The series also included changes of the nutritive state in both directions. Gain of body weight did not necessarily correspond to increase in the pancreas remnant. Also, though the general wasting in emaciation affects the pancreas, the hypertrophy was all the more evident because some of the most marked examples were found in animals which lost a large proportion of their body weight between operation and autopsy.

Character of Tissue.—In certain instances, especially soon after operation, the increase of weight may be due to inflammatory tissue,

TABLE III.

Alterations in the Weight of the Pancreas Remnant after Partial Pancreatectomy.

No. of instances in which weight of pancreas remnant at autopsy was found within \pm 0.4 gm. of estimate at operation	35
No. of instances of atrophy beyond the above limit of error.....	17
Average atrophy of the group (in percentage of estimated weight of remnant).....	27.4 per cent.
Maximum atrophy	45 " "
No. of instances of hypertrophy to less than 150 per cent of the estimated weight...	28
" " " " " " " 150 per cent of the estimated weight.....	30
" " " " " " " 200 " " " " " " "	30
" " " " " " " 300 " " " " " " "	8
" " " " " " " 400 " " " " " " "	4

but this is not the true hypertrophy referred to. Sometimes also fairly normal parenchyma may be distorted or more or less encapsulated by superficial scar tissue. But in the best examples the remnant is free from perceptible sclerosis and consists of lobulated parenchyma normal in appearance and consistency. Microscopic examination confirms the absence of fibrosis. The acini may be particularly large and crammed with secretion. As described for Dogs 148 and 151⁷ islands may be scanty as if the hyperplasia had been limited chiefly to the acinar tissue, or fairly abundant, as if the islands had kept pace with the proliferation of the other structures.

⁷ Allen,² pp. 761, 969.

Size of Remnants.—The absolute size of the remnant is not a determining factor in hypertrophy. The remnants in Dogs 104, 125, 148, 151, and 152, mentioned above, were of various sizes. The later series includes remnants of 8 to 12 gm. which increased to 14 to 22 gm., also a remnant of 0.8 gm. which increased to 2 gm., one of 1.6 gm. which increased to 5.4 gm., and one of 1.8 gm. which increased to 6.5 gm. Correspondingly, the relative size of the remnants did not govern hypertrophy. Some of the larger remnants referred to were as much as $\frac{1}{4}$ of the pancreas, while the 0.8 gm. remnant mentioned was only $\frac{1}{18}$ of the total organ.

Relation to Diabetes.—It follows from the last statement that diabetes was not a determining factor in hypertrophy, which was present or absent in diabetic and non-diabetic animals indiscriminately. In other words, the stimulus did not apparently originate from lack of either the internal or external function of the pancreas. A still more important question is to what extent the hypertrophy was able to prevent or cure diabetes. In some instances the new formed tissue appeared functionally equivalent to the old, for not only was diabetes checked, but also to reproduce it a further resection of tissue was necessary to reduce the remnant to the size requisite in original operations. On the other hand, diabetes sometimes stopped when there was little or no hypertrophy of the remnant; also hypertrophy was frequent with continuance of diabetes. Closer study of these results, which appear contradictory at first glance, brings out the following explanatory facts. (a) The traumatic inflammation following operation is generally a factor in producing diabetes in cases with fairly large remnants. When by any means symptoms are prevented until the inflammation has subsided, it is sometimes found that the animal is no longer diabetic even though there has been little or no increase in the size of the remnant. (b) Inflammation may injure chiefly the islands, to such an extent that the animal remains diabetic after the inflammation has subsided notwithstanding hypertrophy of the remnant. If a functional injury may be assumed, as seems to be true in human diabetes, an explanation is afforded of the existence of diabetes even when the hypertrophic remnant contains numerous islands, as in Dog 151,⁶ but this point is still doubtful. (c) Overfeeding furnishes the most

frequent explanation, and may be put to valuable use. For example, severe acidosis is obtainable only when the dogs can digest large quantities of fat, and dogs with small pancreas remnants can seldom do this. But by cautious overfeeding with carbohydrate after operation, mild diabetes may be kept up, while in a certain proportion of animals marked hypertrophy of the remnant is taking place. Islands are thus injured or destroyed, while the great increase of acinar tissue enables a satisfactory digestion of fat.

Age of Animals.—The animals mentioned above were fairly young, mostly 2 or 3 years of age. But both hypertrophy and its absence have been noted in dogs at all ages. Dog 146⁷ was senile, yet the pancreas remnant in 3 weeks increased from 2.8 gm. to 6.3 gm. Also the records of puppies in a subsequent paper indicate no remarkable regenerative activity, just as the damaged pancreas of diabetic children fails to recover to any greater extent than that of adults.

Time Element.—It is impossible to determine by weight the beginning of hypertrophy, because the operation ordinarily is followed by inflammatory exudate and infiltration. Epithelial hyperplasia begins almost simultaneously and sometimes proceeds with astonishing rapidity. The most rapid increase observed was in Dog 152; namely, the growth of an estimated 4.4 gm. remnant to 10.4 gm. in 11 days. The hypertrophy seems to be mostly complete within the first few weeks after operation. The result is permanent, as shown in observations covering 3 years and over.

Cause of Hypertrophy.—The occasional hypertrophy of a mass of pancreatic tissue to several times its size, in contrast to the relatively slight increase in the great majority of instances, seemed a phenomenon worthy of attention for possible application in the treatment of human diabetes. Various attempts, by trimming remnants in different shapes, by multiple incisions through the surface, and by leaving protruding ducts as possible origins of proliferation, gave no consistent results. Here, as in certain of the preceding paragraphs, it is necessary to anticipate some of the findings of the microscopic study. The recently traumatized tissue shows a mass of inflammatory, degenerative, and regenerative changes, and it is inferred that the predominance of any of the three determines the result.

There is no warrant for attempting to stimulate regeneration of the human pancreas by mere trauma. Some more hopeful method may ultimately be worked out. The observations are important as confirmation of other evidence of the regenerative power of the pancreas in postembryonic life.

Repeated Operations.

In Table III, it was possible only to pick out the maximum weight of the pancreas remnant with which different animals developed diabetes in single operations. Each animal in Table IV was subjected to successive removals of pancreatic tissue, so that the exact reduction of the organ requisite for diabetes was sometimes determined within a fraction of a gram. The results in general confirm those of Table III. The traumatic inflammation of repeated operations accounts for the occasional occurrence of diabetes with large remnants. The degree of hypertrophy, obtained as a total by adding together the weights of tissue removed in operations and present at autopsy, was similar to that observed with single operations.

The observations were mostly long, generally continuing for months or years after the last operation. The operations were from two to five in number, and the intervals between them ranged from 2 weeks to 32 months. The time elapsed made no demonstrable difference in the susceptibility to diabetes. This conclusion has reference to the suggestion of some former writers that animals may develop "immunity" to the lack of pancreatic tissue, particularly by vicarious action of other glands. Such ideas are probably explained by the fact that these workers dealt with atrophic pancreas remnants lacking duct communication with the bowel, so that (*a*) in subsequent operations some fragments were missed, and (*b*) cachexia hindered the development of typical diabetes.

The same observation supports the conclusion of papers to follow that partially depancreatized animals show no inherent increase of tendency to diabetes with time.

TABLE IV.

Repeated Operations.

Dog No.	Normal body weight.	Total pan- creas weight.	Remnant.		Hyper- troph.	Time elapsed between first and last opera- tions.	Remarks.
			Esti- mated weight.	Frac- tion.			
	kg.	gm.	gm.		gm.	mos.	
B2-02	10.5	30.5	6.8	$\frac{1}{4} - \frac{1}{8}$		32	Very mild diabetes, due to traumatic inflammation.
B2-05	6.5	11.5	1.2	$\frac{1}{8} - \frac{1}{8}$		$\frac{1}{2}$	Mild diabetes, stopped by dis-temper.
B2-43	11.1	18.8	5.1	$\frac{1}{4}$	5.1-5.8	$\frac{3}{4}$	Slow onset of mild diabetes.
B2-51	9.75	26.2	2.75	$\frac{1}{9} - \frac{1}{10}$		1	Severe diabetes.
B2-53	10.25	24.5	3.5	$\frac{1}{2}$	3.5-4.5	$\frac{3}{8}$	Cachexia.
B2-63	24.4	49.0	7.4	$\frac{1}{6} - \frac{1}{7}$	7.4-14.2	22	Diabetes resulted from subse-quent operations removing 1.32, 0.36, and 0.22 gm.
B2-88	14.4	30.7	2.8	$\frac{1}{11}$	2.75-2.75	$1\frac{1}{2}$	Subcutaneous graft also pres-ent. Diabetes followed its removal.
B2-89	13.1	30.1	2.7	$\frac{1}{11}$		11	Diabetes checked by emacia-tion; restored by removal of additional 0.65 gm.
C3-20	24.0	51.9	11.5	$\frac{1}{4} - \frac{1}{8}$	11.5-20.2	$4\frac{1}{2}$	Mild diabetes after four subse-quent operations removing total of 5.3 gm. Remnant at autopsy weighed 14.9 gm.
C3-27	16.25	38.4	4.4	$\frac{1}{9}$	4.4-8.7	8	Severe diabetes after removal of additional 0.9 gm.
C3-45	10.8	27.5	3.7	$\frac{1}{7} - \frac{1}{8}$		$4\frac{1}{4}$	Transitory diabetes; permanent after removal of additional 0.3 gm.
C3-58	15.9	43.8	2.9	$\frac{1}{16}$	2.9-4.25	$4\frac{1}{2}$	Diabetes controlled by diet. Dog emaciated to 9 kilos.
C3-86	15.0	35.9	2.9	$\frac{1}{12} - \frac{1}{13}$	2.9-1.8	$7\frac{1}{2}$	Anorexia prevented diabetes till additional 0.2 gm. was removed. Sclerosis and atro-phy of remnant.
C3-98	13.4	27.9	2.1	$\frac{1}{13}$	2.1-4.4	8	Diabetes controlled by diet. Later two operations re-moving total of 1.45 gm. necessary to bring back diabetes.
D4-63	15.25- 18.7	46.5	4.6*	$\frac{1}{10}$		$7\frac{2}{3}$	Mild diabetes.
D4-85	10.0	25.9	2.0	$\frac{1}{13}$		1	Severe "
D4-86	15.9	35.5	3.9	$\frac{1}{9}$	3.9-10.8	$1\frac{1}{2}$	" "
D4-90	36.9	82.8	1.9	$\frac{1}{42} - \frac{1}{43}$		1	" "
D4-96	11.6	24.2	1.8	$\frac{1}{13} - \frac{1}{14}$	1.8-2.4	$3\frac{2}{3}$	" "
E5-00	15.25	35.0	3.5	$\frac{1}{10}$		$\frac{2}{3}$	" "
E5-16	18.0	34.0	6.75	$\frac{1}{2}$	6.75-9.0	$\frac{2}{3}$	Mild "

*Weight at autopsy.

Pancreas Weight in Relation to Reduced Body Weight.

The filling of the acini with zymogen during short fasts and the discharge on functional stimulation are probably sufficient to cause considerable changes in the gross weight. On the other hand, long fasting is accompanied by shrinking of the acinar cells and diminution

TABLE V.
Dogs with Malnutrition.

Dog No.	Normal body weight.	Final body weight.	Pan- creas weight.	Weight of pan- creas per kilo of normal body weight.	Weight of pan- creas per kilo of final body weight.	Remarks.
	kg.	kg.	gm.	gm.	gm.	
C3-17	12.6	10.5	22.2	1.76	2.11	Poor appetite for 3 wks. 110 gm. of glucose subcutaneously 3 hrs. before death.
D4-27	12.5	8.8	22.1	1.77	2.51	Overfatty diet for 3 mos. Fat intoxication. 40 gm. of glucose subcutaneously 6 hrs. before death.
D4-31	10.6	8.0	12.1	1.14	1.51	Fasting for 2 wks., followed by overfatty diet for 6 wks. Fat intoxication.
F6-54		5.6	24.9		4.45	Thin when received. Killed with phosphoric acid intravenously.
F6-95	4.3	3.7	8.5	1.98	2.30	Fasting for 12 days; poor appetite following 12 days. Death from acetoacetic acid intravenously.
G7-21		15.0	30.6		2.04	Very thin when received.
G7-87		6.4	14.7		2.30	" " " " Killed with bicarbonate intravenously.
G7-89	18.0	14.0	47.8	2.66	3.41	Fasting and phlorizin 1 wk.; appetite poor 1 wk.; then killed with acid and alkali intravenously.
G7-99		8.0	15.0		1.87	Thin when received. Death from shock and bicarbonate.
C3-01		14.9	29.6		1.99	Collie; very thin when received.

of zymogen, so that in some cases the pancreas consists entirely of involuted rounded cells, with only rare traces of zymogen and little or no visible acinar arrangement.⁸ The gross organ is softer and smoother, less lobulated, sometimes almost translucent or gelatinous

⁸ Allen,² Chapter XXI, also Figs. 2, 3, and 4.

in areas, and obviously below normal weight. The present series did not include extremely short or long fasts, but only such intermediate degrees of abstinence or reduced diet as caused appreciable reduction of body weight.

Table V is made up of dogs which were either received in a very emaciated condition or suffered more or less loss of weight under

TABLE VI.

Fasting Dogs.

Dog No.	Normal body weight.	Final body weight.	Pan- creas weight.	Weight of pan- creas per kilo of normal body weight.	Weight of pan- creas per kilo of final body weight.	Remarks.
	kg.	kg.	gm.	gm.	gm.	
C3-15	14.5		17.3	1.19		Long voluntary fast. 150 gm. of glucose by stomach 1½ hrs. before death.
D4-01	14.5	10.6	22.8	1.57	2.15	Undernutrition for 54 days, followed by fasting for 4 days.
F6-50	6.0	4.2	16.7	2.78	3.98	Fasting for 11 days with occasional doses of hydrochloric acid.
F6-52	6.3	5.0	13.4	2.13	2.68	Fasting for 8 days. Killed with acid sodium phosphate intravenously.
F6-79	10.2		23.1	2.26		Fasting for 20 days with large doses of hydrochloric acid.
F6-99	6.4	4.5	12.2	1.91	2.71	Fasting for 17 days. Death from 20 gm. of lactose intravenously.
G7-00	5.5	3.5	13.6	2.47	3.89	Fasting for 25 days. 25 gm. of glucose intravenously on last day. Milk vomited.
G7-01	5.5	4.4	10.3	1.87	2.34	Fasting for 18 days. Death from dextrin intravenously.
B2-52	12.8	9.9	23.5	1.84	2.38	Fasting for 16 days before operation.
B2-57	11.1	8.6	21.6	1.95	2.51	" " 18 " " "
B2-58	11.8	9.1	18.0	1.53	1.98	" " 18 " " "

observation. The animals of Table VI underwent actual fasting for various periods, sometimes together with other experimental procedures as noted. Those of Table VII received phlorizin as stated, subcutaneously, besides the fasting or undernutrition imposed.

In a majority of instances, both the absolute and relative weights of the pancreas fall within the normal extremes shown in Table II,

TABLE VII.

Fasting or Undernutrition with Phlorizin.

Dog No.	Normal body weight.	Final body weight.	Pan- creas weight.	Weight of pan- creas per kilo of normal body weight.	Weight of pan- creas per kilo of final body weight.	Remarks.
	kg.	kg.	gm.	gm.	gm.	
C3-54	19.5	15.0	20.5	1.05	1.37	Fasting with 1 gm. of phlorizin daily for 10 days. Lard feeding and bicarbonate intravenously on day of death.
C3-57	14.5		13.9	0.96		Fasting for 9 days; pure fat feeding on last 3 days. 1 gm. of phlorizin daily.
C3-69	17.5	13.9	23.0	1.31	1.65	Undernutrition, followed by fasting for 5 days with 1 gm. of phlorizin daily. Glucose intravenously on day of death.
C3-99	22.5	17.0	31.2	1.39	1.83	Protein-fat diet with 0.5 or 1 gm. of phlorizin daily for 50 days.
D4-00	13.2	10.8	17.9	1.36	1.66	Protein-fat diet with 0.5-0.75 gm. of phlorizin every day or two for 50 days. Glucose and bicarbonate on day of death.
E5-07	15.0	11.4	30.8	2.05	2.70	Fasting for 10 days with four doses of 1 gm. of phlorizin. 50 gm. of glucose by stomach 2 days before death.
E5-33	17.5	13.9	35.0	2.00	2.52	Fasting for 1 wk. with five doses of 0.5 gm. of phlorizin.
F6-22	13.6	9.8	31.7	2.33	3.24	Fasting for 10 days with 0.5 gm. of phlorizin daily. 50 gm. of glucose subcutaneously on day before death.
F6-25	12.0	7.1	18.5	1.54	2.63	Fasting for 11 days with three doses of 0.5 gm. of phlorizin.
F6-32	17.6		27.0	1.53		Fasting for 6 days with three doses of 1 gm. of phlorizin. Bicarbonate on last 2 days.
F6-33	14.2		22.7	1.60		Fasting for 9 days. Bicarbonate on 2 days before death, and sodium chloride on 2 days before that.
F6-67	16.0	11.0	31.1	1.94	2.83	Fasting for 16 days with seven doses of 1 gm. of phlorizin.
F6-91	12.7	9.0	20.3	1.60	2.26	Partial nephrectomy. Fasting for 15 days with four doses of 1 gm. of phlorizin. Fed meat on 11th day of fast. 25 gm. of glucose subcutaneously on 13th day.

TABLE VII—*Concluded.*

Dog No.	Normal body weight.	Final body weight.	Pan-creas weight.	Weight of pan-creas per kilo of normal body weight.	Weight of pan-creas per kilo of final body weight.	Remarks.
	kg.	kg.	gm.	gm.	gm.	
G7-28	4.5		10.9	2.42		Fasting for 5 days with three doses of 0.5 gm. of phlorizin. Butyric and aceto-acetic acids intravenously.
G7-75	12.6	11.5	18.5	1.47	1.61	Fasting for 5 days with two doses of 1 gm. of phlorizin.
G7-76	11.2	9.0	20.4	1.82	2.26	Fasting for 8 days with four doses of 1 gm. of phlorizin. Early pregnancy and abortion.
G7-82	15.0		24.8	1.65		Fasting for 7 days with three doses of 1 gm. of phlorizin.
G7-93		11.4	23.7		2.08	Fasting for 4 days with one dose of 0.5 gm. of phlorizin on 1st day.
G7-96	11.0	10.0	25.5	2.32	2.55	Fasting for 5 days with two doses of 0.5 gm. of phlorizin.

whether calculated on the normal or the reduced body weight. Also in a majority of instances, they are closer to the average for the reduced than for the normal body weight. The rule has such marked exceptions in both directions that a table of averages could not properly be used. The degree of undernutrition is as great as any employed for controlling even the severest diabetes, and the results do not warrant a conclusion that the therapeutic effects of undernutrition are explainable by an increase of the ratio of pancreas mass to body mass. The islands perhaps shrink less than the acinar tissue in fasting, but there is no basis for a conclusion concerning mass relations.

Any marked changes in the mass of the pancreas due to accompanying experiments would also be revealed by such comparisons. For example, the exceptionally large pancreas weights of Dogs F6-54 and G7-89 in Table V represent the edema of the pancreas sometimes produced by acid injections. The other experimental procedures, such as glucose injections and especially phlorizin poisoning, did not demonstrably alter the gross weight of the pancreas.

EXPERIMENTAL STUDIES ON DIABETES.

SERIES I. PRODUCTION AND CONTROL OF DIABETES IN THE DOG.

2. EFFECTS OF CARBOHYDRATE DIETS.

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The belief underlying the dietetic treatment of diabetes has been that the disorder consists in the weakness of a bodily function, which is broken down by overstrain and spared or strengthened by rest. A coexistent and equally firmly rooted belief has been that some or perhaps most human cases are inherently progressive, on account of continuing toxic, infectious, or undetermined factors, so that they become worse and lead to death after a longer or shorter time in spite of dietary restrictions. Attention was previously directed¹ to the importance of determining this point positively in dogs, which are free from constitutional tendencies. This fundamental question of the possibilities and limitations of injuring assimilation and shortening life by overfeeding and improving tolerance and lengthening life by restricted diet was not fully settled by the experiments of Thiroloix and Jacob² or the present writer, and papers to follow will be devoted particularly to this subject. Microscopic changes will be mentioned briefly here and treated more completely later.

Effect of Starch-Rich Diet.

Dog B2-65.—Female; mongrel; black, slightly shaggy; age 5 years; rather thin; weight 17.5 kilos. May 25, 1914. Removal of pancreatic tissue weighing 28.9 gm.³ Remnant about main duct estimated at 3.4 gm. ($\frac{1}{9} - \frac{1}{10}$). The urine

¹ Allen, F. M., Studies concerning glycosuria and diabetes, Cambridge, 1913, Chapter X.

² Thiroloix, J., and Jacob, *Bull. et mém. Soc. méd. hôp. Paris*, 1910, xxix, series 3, 492; xxx, series 3, 29, 656.

³ All operations were performed under ether anesthesia.

remained sugar-free on fasting till May 29. Bread and soup diet was then begun, and glycosuria continued absent till June 2, after which date it was continuous. Bits of pancreatic tissue were removed by operations on June 15 and July 9 without apparently altering the steady downward progress. Glycosuria increased; at the outset it was as low as 1.5 per cent in 200 cc. of urine for 24 hours, at the end as high as 9.1 per cent in 2,415 cc. of urine. From July 23 to 28, the sugar was only once below 14 per cent, being thus exceptionally high in percentage. The animal remained in good spirits, but the weight diminished to 9 kilos, with corresponding loss of strength. Death occurred from weakness on July 29.

Autopsy.—Except for the emaciation, the autopsy was entirely negative. The pancreas remnant weighed 3.35 gm. The samples of pancreatic tissue for June 15, July 9, and autopsy showed normal acini and successive stages of hydropic degeneration of islands, involving finally a marked reduction in number and size of islands but by no means complete disappearance.

This record may be considered typical of the rapidly fatal course of this form of experimental diabetes on starch-rich diet, corresponding to the rapid emaciation and death in the severest human cases when entirely untreated, though the process is generally more rapid in dogs than in patients.

Breaking Down Tolerance with Glucose.

Dog B2-31.—Female; bull-terrier; white; age 2 years; good condition; weight 11 kilos. Dec. 23, 1913. Removal of pancreatic tissue weighing 19 gm. Remnant about main duct estimated at 2.6 gm. ($\frac{1}{8}$ – $\frac{1}{4}$). Subsequently, bread feeding caused only a fraction of a per cent of glycosuria, which ceased and remained absent even on addition of glucose, until the quantity of glucose reached 200 gm. daily. Glycosuria ranging from 2 to 5.5 per cent in the mixed 24 hour urine of each day continued on the diet of bread and soup with glucose as stated till Feb. 12, 1914. The diet was then changed to meat *ad libitum*. Glycosuria steadily diminished during the succeeding days, till on Feb. 20 it was only 0.22 per cent and was evidently on the point of disappearing. The bread and glucose diet was repeated, with heavier glycosuria than before (up to 5.8 per cent in 24 hour urine of 2,470 cc.), until Feb. 26. The diet of meat *ad libitum* was then resumed, with the result that glycosuria fell as low as 0.9 per cent in 367 cc. of urine on Feb. 28, but rose to 6 per cent in 1,090 cc. of urine on Mar. 6. The condition thereafter was severe diabetes, and the animal was used for therapeutic tests, stringent regulation of diet and weight being necessary to keep glycosuria absent.

This animal was specially suited for the purpose because of an unusually voracious and unfailing appetite and a natural fondness for sweets. Most dogs will not eat so much bread and sugar for so long

a time. Animals and patients alike can evidently bring on diabetes by gluttony, particularly in starch and sugar, when a certain degree of predisposition exists.

Indigestion and diarrhea check the carbohydrate excess and prevent diabetes in many animals, and strong digestive power facilitates the production of experimental and doubtless also of clinical diabetes. Talcum powder mixed with the food, in quantities up to 100 gm. or more daily, has been the chief means used to control diarrhea. The innocuousness of talcum in any amount or duration for such experiments suggested its introduction later as a flour for making pancakes for some patients on extremely low diets.

The downward progress of human diabetes can be further imitated in that the disease in dogs can be made slow or rapid as desired by varying the kind and quantity of food. Like human patients these dogs never regain a tolerance for food equal to that which they possessed before the damage from overfeeding occurred.

The plan of breaking down tolerance with the largest feasible quantities of starch and glucose immediately after operation is of practical importance for securing diabetic dogs with the largest possible pancreas remnants, and hence with the best possible digestive power and vigor. Knowledge of animals and skill in managing their diets are important for success in producing the most valuable test objects for diabetic research; namely, dogs which eat and digest well and appear normal in all respects except for the desired degree of diabetes. The best results require that the investigator should give personal attention to this work.

Dog B2-80.—Female; mongrel; black and white; age 3 years; very good condition; weight 17 kilos. Nov. 17, 1914. Removal of pancreatic tissue weighing 25.7 gm. Remnant communicating with both ducts estimated at 7.1 gm. ($\frac{1}{4}$ – $\frac{1}{2}$). The dog was specially valuable, because with this large pancreas remnant immediately after operation she had a tendency to slight glycosuria on plain bread and soup diet, and was easily kept glycosuric by the addition of small quantities of glucose. After a period of sugar freedom on meat diet, the dog was loaned to another laboratory, and was returned with the statement that she could not be made diabetic. Trial with increasing dosage of glucose showed that glycosuria actually remained absent with such quantities as could voluntarily be eaten, and there was danger here as in many cases with large remnants that spontaneous recovery of assimilation due to the delay might spoil the opportunity for diabetes.

Accordingly, beginning Apr. 24, 1915, 500 gm. of glucose were given daily. Diarrhea was controlled by the use of bone-meal and talcum powder, and any portions of the sweetened bread and soup mixture left uneaten were made into firm balls and fed forcibly. Only a 5 day period of this excessive dosage was necessary before it became possible to reduce the quantities with continuance of glycosuria. The animal was later overfed with protein and fat so as to bring on severe diabetes and acidosis. Aug. 14. Died in coma.

Autopsy.—The dog possessed abundant fat at death, like some human patients dying of acidosis. The body weight was 17.25 kilos, the weight of the pancreas remnant 13.5 gm. Its tissue was soft, normally lobulated and free from fibrosis, but microscopically consisted of unbroken expanses of acini. Rare small clumps, apparently of alpha cells, were the only remains of islands. Many small ducts showed appearances like vacuolation.

Unsuccessful Attempts to Break Down Tolerance.

Dog B2-02.—Female; Boston terrier; brindle; age 1½ years; weight 10.5 kilos. This dog was received Oct. 25, 1913, and was used for various experiments, especially the removal of successive portions of pancreas tissue, until an operation on Dec. 10, 1914 at length produced diabetes. Glycosuria was present up to Dec. 23 on plain bread and soup feeding. It could readily be produced thereafter by the addition of glucose. But the dog was exceptionally dainty in appetite, would eat only limited quantities of bread, had a strong distaste for sugar, and vomited readily and persistently if forcibly fed. The potential diabetes continued, as proved by the easy production of glycosuria by occasional glucose feedings and the low tolerance for glucose subcutaneously, but active symptoms could never be maintained. Aug. 31, 1915. A bit of pancreas tissue was removed for examination. The condition thereafter was unchanged, namely absence of glycosuria with bread diet, also with the addition of 100 gm. of glucose, but glycosuria with the addition of 200 gm. of glucose; and all such attempts were terminated within a few days by refusal and vomiting. On mixed diet of bread and meat the dog continued in full health at the original weight of 10.5 kilos, until death from rabies, Sept. 19, 1916.

Of numerous animals in which the determining influence of appetite upon the occurrence of diabetes was observed, this one afforded the most striking antithesis to Dog B2-31 above mentioned. The actual assimilative power was much lower, in consequence of operation, in this dog than in Dog B2-31, and the eating habits were alone responsible for the opposite results.

Autopsy.—The pancreas remnant weighed 6.8 gm. Grossly it was normal in appearance and consistency, and microscopically the acini were fully normal; but occasional small fibrous trabeculae and a general scarcity of islands indicated a previous inflammation as the probable cause of the potential diabetes with such a large remnant of pancreatic tissue. Also the island cells, both on Aug. 31, 1915, and at autopsy, showed numerous suggestions of vacuolation, generally so slight

as to be doubtful. These anatomic findings are of importance in connection with the question discussed below of the probable ultimate outcome of such a diet in such an animal.

Dog B2-86.—Male; short haired mongrel; cinnamon and black; very old, obese, and phlegmatic, but vigorous and active; weight 32.4 kilos. Apr. 7, 1914. Removal of pancreatic tissue weighing 48.9 gm. Remnant about main duct estimated at 12.4 gm. ($\frac{1}{8}$). The dog was inordinately greedy, but had no glycosuria even from the largest amounts of bread and soup which he ate. Apr. 19. This diet was increased by 250 gm. of glucose, with resultant glycosuria of 1.25 per cent, which steadily diminished to 0.2 per cent on Apr. 23. The glucose was then increased to 500 gm. daily, but nevertheless glycosuria was absent thereafter. It was possible to continue heavy glucose feeding with only a few days of intermission to avoid anorexia, indigestion, or diarrhea. The bread was ordinarily not weighed, but on certain test days the dog eagerly ate as much as 1 kilo of dry bread and 800 gm. of glucose. The average bread ration was nearer 500 gm., but 400 to 600 gm. of weighed glucose were taken with this. No other dog in the series was willing to eat or able to digest such quantities of carbohydrate, and advantage was taken of this peculiarity in order to test whether diabetes might ultimately result from such a load upon the carbohydrate metabolism. Glycosuria was rare and slight. Gain in tolerance was also indicated by tests on May 4 and 13. On each of these dates the dog was fed 400 gm. of bread, 200 gm. of glucose, and 500 cc. of soup, and the urine and plasma sugars were followed as shown in the accompanying table.

Dog B2-86.

Date.	Time.	Plasma sugar.	Urine.		Date.	Plasma sugar.	Urine.	
			Volume.	Sugar.			Volume.	Sugar.
1914		per cent	cc.	per cent	1914	per cent	cc.	per cent
May 4	Before feeding.	0.089	376	0	May 13	0.099	123	0
	$\frac{1}{2}$ hr. after feeding.	0.196	12	Faint.		0.196	6	0
	1 $\frac{1}{2}$ hrs. " "	0.286	15	0.52		0.156	5	0
	2 $\frac{1}{2}$ " " "	0.298	21	0.52		0.192	2 drops	Faint.
	3 $\frac{1}{2}$ " " "	0.357	14	1.76		0.232	25	0.62
	4 $\frac{1}{2}$ " " "	0.476	80	3.53				
	6 " " "	0.435	75	3.61		0.285	25	1.22
	8 " " "	0.400	95	3.22		0.270	25	1.13
	9 " " "	0.270	55	2.22		0.286	35	1.00
	11 " " "	0.294	45	1.81		0.250	75	0.55
	13 " " "	0.263	3	0.96				
	14 " " "	0.156	100	0.70		0.176	110	Faint.
	16 " " "	0.099	40	0		0.098	155	0
	24 " " "	0.062	143	0		0.098	320	0

The poor prospect of overcoming the increased tolerance by higher doses of carbohydrate was indicated by a shorter test on July 21.

Dog B2-86.

Date.	Time.	Plasma sugar.	Urine sugar.	Remarks.
1914		<i>per cent</i>	<i>per cent</i>	
July 21	Before feeding.	0.124	0	Fed 400 gm. of bread with 500 gm. of glucose.
	2 hrs. after feeding.	0.147	0	
	7 " " "	0.141	0	

Aug. 7. Removal of a bit of pancreas was attempted. The remnant was excessively congested; stubborn oozing prolonged the operation a little, and the dog died under the operation. The pancreatic congestion was not necessarily associated with the sugar feeding, for it has been found in occasional dogs under other conditions. The easy death from ether or shock may have been due to injury from the glucose, though the animal seemed as well and strong as ever, or merely to senility and obesity.

Autopsy.—The pancreas remnant weighed 24.8 gm. and the tissue including the islands was normal in all respects. The hypertrophy of the remnant was thus accompanied by recovery from diabetes to such an extent that no apparent injury resulted from the most excessive gluttony in carbohydrate for 4 months. The intact state of the islands was evidence that more prolonged carbohydrate excess could not have caused a lowering of tolerance, which is due to hydropic degeneration of the islands as formerly described.

Repeated Operations.

It is a familiar and readily comprehensible experience that glycosuria may be absent or transitory on the highest possible starch and sugar feeding, and that the diabetes which could not be induced by diet is brought on by the removal of an additional tiny fragment of pancreas. An example was previously given.⁴ Others are mentioned in the preceding paper.⁵ It is there seen that 0.3 gm. of tissue in the case of Dog C3-45, or 0.22 gm. in the final operation of Dog B2-63, sufficed to make the difference between diabetes and its absence. Certain points will be further developed by giving a summary of individual protocols.

⁴ Allen,¹ p. 486, Dog 176.

⁵ Allen, F. M., *J. Exp. Med.*, 1920, xxxi, 375, "Remarks" column of Table IV.

Dog B2-63.—Male; mongrel; brown and white, shaggy; age 6 years; slightly thin; weight 24.4 kilos. May 22, 1914. Removal of pancreatic tissue weighing 41.6 gm. Remnant about main duct estimated at 7.4 gm. ($\frac{1}{8}$ – $\frac{1}{4}$). Glycosuria was continuously absent on a diet of beef lung up to June 23. The diet was then changed abruptly to bread and soup. Glycosuria of 0.2 per cent in 1,140 cc. of urine was present on June 24, but ceased immediately. June 29. 100 gm. of glucose were added, with resultant glycosuria of 0.52 per cent in 1,185 cc. of urine; this also ceased immediately. Similar transitory traces of glycosuria followed the increase to 200 gm. of glucose on July 1, to 300 gm. on July 8, and to 400 gm. on July 30. On Aug. 3 (after more than a month of continuous bread and glucose diet) the attempt to produce diabetes by feeding was abandoned, and the dog was used for phlorizin experiments.

Nov. 6. An additional 1.32 gm. of pancreatic tissue was removed, the body weight being then 23.9 kilos. Nov. 23. The urine was still sugar-free on bread and soup diet; 100 gm. of glucose were given by stomach tube before feeding. This procedure caused moderate glycosuria on this day, less on the next day, and none on the following days. Nov. 28. The glucose feeding was stopped. Thereafter on plain bread and soup diet, slight to moderate sugar reactions appeared on Dec. 1, 8, 9, 10, 12, and thenceforth continuously to Jan. 3, 1915. The occurrence of glycosuria at first was governed by the quantities of bread eaten, the dog being hungrier on certain days. This lowering of tolerance on prolonged carbohydrate diet took place notwithstanding a loss of body weight to 22.7 kilos on Dec. 12. Glycosuria ceased with a single fast day on Jan. 3, and thereafter was continuously absent on a diet of beef lung *ad libitum*. Mar. 30. Weight 22 kilos; the only food given was 200 gm. of bread (weighed dry, and moistened with soup before feeding). The result was glycosuria of 0.87 per cent in 860 cc. of urine. Several other tests as mentioned below, to compare the assimilation of different starches, indicated a similar lowering of tolerance. As the dog did not thrive on the monotonous lung diet, the weight gradually fell, reaching its lowest level of 17.9 kilos on June 26. Corresponding to this marked decline in weight was a rise in tolerance, so that 200 gm. of bread no longer caused glycosuria; also on July 6, at a body weight of 18.75 kilos, the sugar in the blood plasma after ingestion of this quantity of bread rose only to 0.143 per cent. The diet was then changed to lung and suet *ad libitum*; the body weight gradually rose to 27 kilos in Aug. and Sept. The dog proved able to take 200 gm. of bread in addition to the usual diet, and on Sept. 20 the diet was changed to bread and soup with suet. Glycosuria remained absent, with the exception of 0.34 per cent on Oct. 7 and 0.32 per cent on Oct. 10. The body weight gradually rose, until by the middle of Nov. the animal was obese at a weight of 30.9 kilos. Nov. 16. Slight glycosuria appeared, and increased on Nov. 17. With 1 day of fasting and 1 day of exercise, glycosuria ceased and remained absent, on a diet of bread and soup, while the body weight fell to 30 kilos.

Nov. 30. Weight 30 kilos; pancreatic tissue weighing 0.36 gm. was removed. During the ensuing week traces of glycosuria occurred on bread and soup diet, though the dog ate poorly. Fasting was then imposed from Dec. 7 to 16.

Dec. 16. Weight 24.25 kilos. The animal was given all the bread and soup he would eat. Heavy glycosuria appeared (hunger glycosuria), diminishing to faint glycosuria on the following day and remaining absent thereafter, except for a trace of 0.14 per cent on Dec. 31. The weight slowly fell to 23 kilos by the middle of Feb.

Beginning on Feb. 14, 1916, at this weight, 200 gm. of glucose were added to the bread and soup diet. On this day there was glycosuria of 0.37 per cent, which ceased immediately. Beginning Feb. 18, 100 gm. of lard were added daily to the bread and glucose diet. The weight rose to 24.7 kilos by Feb. 26, while glycosuria remained absent. On this date the dog was changed from the warm room to an outdoor cage. On the identical diet traces of glycosuria were continuous for a few days, as will be described in a subsequent paper. The weight was meanwhile falling to 23.4 kilos, and on Mar. 4 to 6 an increase to 400 gm. of glucose produced no more than 0.45 per cent glycosuria in 600 cc. of urine.

Mar. 7. Pancreatic tissue weighing 0.22 gm. was removed. Thereafter bread and glucose feeding maintained glycosuria without difficulty. Mar. 27. An additional 0.8 gm. of pancreatic tissue was removed. Apr. 2. Death occurred from peritonitis.

Autopsy.—The pancreas remnant weighed 12.3 gm.

Microscopically, the important feature was the existence of vacuolation of island cells throughout the series of pancreatic specimens, very slight and scarce in the operations of Nov. 6, 1914, Nov. 30, 1915, and Mar. 7, 1916, and decidedly more advanced on Mar. 27, 1916, and at autopsy.

The clinical course, especially with consideration of the shorter life of a dog, bears a resemblance to a prolonged mild case of human diabetes. The vicissitudes and fluctuations of tolerance might appear superficially as bizarre and accidental, but comparison with numerous other examples furnishes a rational explanation for each change.

The factors to be observed particularly as influencing the tolerance are the diet, the body weight, and the natural reparative power. In brief, it will be noticed that up to January, 1915, heavy carbohydrate feeding reduced the tolerance, even though the urine was generally sugar-free and even though the body weight fell. This lowering of assimilation was still perceptible on March 30, but with carbohydrate-free diet and further decline of weight, the tolerance in June and July was found higher, and in September the animal was able to live on bread without glycosuria. The body weight was then increased by

the addition of fat to the diet. Evidence of actual reparative power in the pancreas was afforded, for the improved assimilation continued even at higher weights than before. But when in November the animal became obese at a weight above 30 kilos, decided glycosuria appeared. The same combination is evident in the operation of November 30 and the subsequent fast. The loss of 0.36 gm. of pancreatic tissue was more than counterbalanced by the reduction to 24.25 kilos in weight; but the genuineness of the reparative process is proved by the higher tolerance and weight as compared with the preceding March.

The reparative process mentioned may be associated with the marked hypertrophy of the pancreas remnant. The recovery, however, was overbalanced by the subsequent removal of 0.36 and 0.22 gm. of additional pancreatic tissue. The newly formed tissue therefore could not be functionally equivalent to the original tissue.

Even though no obvious clinical injury seemed associated with the carbohydrate excess preceding November 6, 1914, or with the obesity of November, 1915, or with the carbohydrate excess prior to March 7, 1916, the microscopic examination demonstrated injuriously overtaxed island cells at all these times. The downward progress clinically would doubtless have been still more evident if it had not been checked at certain periods as mentioned, and also if the observations had been extended for a still longer time. This downward progress is fully comparable with that of mild human cases treated on the lax plan of merely keeping the urine sugar-free most of the time, and it cannot properly be called spontaneous.

It will bear repeating that experiments of this sort require prolonged careful attention to the diet and all other details. Care must be taken that the animal likes his food and eats it, that he does not suffer from diarrhea or other illness, and that the regularity of his program shall equal that of diabetic patients whom he is supposed to imitate. Even so, only a minority of dogs thrive under the conditions and are adaptable to changes of diet and weight as desired. Therefore they are generally obtainable only by trial and choice among a considerable series of dogs. The best ones, which prove capable of such a delicately balanced tolerance and such close reproduction of clinical conditions, represent much labor and are useful for many experiments.

Dog C3-27.—Female; mongrel; yellow; age 4 years; good condition; weight 16.25 kilos. July 8, 1915. Removal of pancreatic tissue weighing 34 gm. Remnant about main duct estimated at 4.4 gm. ($\frac{1}{3}$). The subsequent condition was mild diabetes. Glycosuria was repeatedly produced by feeding 50 or 100 gm. of bread, or on certain occasions by large diets of beef lung. Also at the time of these tests the weight was down to approximately 15 kilos. Beginning Oct. 1, the diet was 500 gm. of beef lung and suet *ad libitum*. Dec. 1 to 5. The addition of 100 gm. of bread caused daily glycosuria not above 0.42 per cent in 450 cc. of urine, the weight then being 19.8 kilos. On omitting the bread the glycosuria ceased. Beginning Feb. 14, 1916, the addition of 100 gm. of bread produced no glycosuria. Feb. 23. The diet was changed to bread and soup. Feb. 25. 100 gm. of glucose were added to this diet, causing glycosuria of 0.63 per cent in 940 cc. of urine for 1 day. Glycosuria then ceased and could not be restored, though the glucose dosage was 200 gm. on Feb. 27, 300 gm. daily from Feb. 28 to Mar. 4, and 400 gm. daily to Mar. 9, when the attempt had to be abandoned because of distaste for the bread and glucose mixture. The dog meanwhile had become obese, reaching the maximum weight of 21 kilos.

Mar. 9. An additional 0.9 gm. of pancreatic tissue was removed. Bread diet caused no glycosuria on the following days. Beginning Mar. 13, 200 gm. of glucose were added daily, with negative urine till Mar. 16, when heavy glycosuria suddenly appeared. This continued after glucose was discontinued on Mar. 21. The subsequent course was downward progress on mixed diet to death in coma on Apr. 22.

Autopsy.—The pancreas remnant consisted of normal appearing tissue weighing 7.8 gm. Islands of Langerhans were very large and abundant but maximally vacuolated.

In the tissue removed at operation on Mar. 9, the islands were free from any visible abnormality.

Genuine recuperation of function in the pancreas remnant was demonstrated by the double evidence of markedly increased carbohydrate tolerance at a higher body weight, up to March 9. In view of the facts that the dog's digestion could not bear further prolongation of the high glucose feeding, that the islands at that time showed no sign of vacuolation, and that the tendency to recovery was so manifest, it is improbable that diabetes could have been brought on by any kind or duration of feeding.

This recovery of function corresponded to a marked hypertrophy of the pancreas remnant, which doubled in size. The great abundance of islands found in numerous slides indicated that they underwent hyperplasia which fully paralleled that of the acini. Nevertheless, the functional recovery was completely negated by the removal of

only 0.9 gm. of additional tissue. The new formed tissue was therefore not equal to the original tissue in functional capacity.

The reason for the recovery of assimilative power, to such an extent that a second operation was required, lay in the long interval of sugar freedom granted to the animal after the first operation. If the pancreas remnant is of the large size which is most desirable, a dog should be subjected to occasional periods of glycosuria to prevent the tolerance from rising too high. Only after the diabetic condition has been thus maintained for months or years can the animal be trusted to remain permanently diabetic without this precaution. The existence of a considerable recuperative power at the outset, and the feebleness or absence of this power in the later stages, correspond closely to the well known facts in human diabetes.

By comparison with numerous other dogs, it may safely be assumed that carbohydrate overfeeding after the first operation would have sent this animal rapidly into severe diabetes. A carbohydrate-free diet, with limited protein so as to keep the urine sugar-free, permitted recovery of tolerance, even though the fat ration was so high as to produce obesity. Provided that a recuperative power exists in the pancreas, such a diet evidently affords opportunity for recovery and is far less harmful than an excessive carbohydrate diet. This fact was abundantly demonstrated by the benefits of the classical treatment in human cases, and is confirmed by similar observations in dogs.

Dog C3-45.—Female; mongrel; yellow; age 4 years; moderately well nourished; weight 10.8 kilos. Nov. 19, 1915. Removal of pancreatic tissue weighing 23.8 gm. Remnant about main duct estimated at 3.7 gm. ($\frac{1}{3}$ – $\frac{1}{4}$). Glycosuria remained absent with fasting up to Nov. 23. Feeding of bread and soup then brought on heavy glycosuria. Nov. 27. The diet was changed to beef lung; glycosuria progressively diminished, and was absent on Nov. 30. Dec. 1. Heavy sugar (4.45 per cent in 380 cc. of urine) returned on bread diet, then stopped after 2 days of lung diet. Dec. 6. The dog was loaned to another laboratory, where she remained for a time on diets which caused little or no glycosuria. Mar. 16, 1916. The dog was sent back with the report that she was not diabetic. On this date the feeding of bread and soup with 300 gm. of glucose caused excretion of 1.6 per cent sugar in 528 cc. of urine; but the appetite and digestion of the dog could not endure continuance of this sugar dosage, and glycosuria therefore remained absent till Mar. 27. The body weight during this time was 11.3 to 11.6 kilos.

Mar. 27. Pancreatic tissue weighing 0.3 gm. was removed. Glycosuria remained absent with fasting for 2 days. Mar. 29. Bread and soup diet caused faint glycosuria, which became heavy (4.4 per cent in 2,000 cc. of urine) on the addition of 50 gm. of glucose. After Apr. 2, it continued heavy on plain bread and soup diet, and the dog was kept in a condition bordering on glycosuria thereafter. Glycosuria was absent on restricted diet, but always returned promptly on feeding bread or too much meat, and there was no further tendency to any marked recovery of tolerance. The animal was sent to another laboratory on July 10.

The noteworthy feature is that the considerable recovery of tolerance in this case was overbalanced by the removal of 0.3 gm. of additional tissue, and that this tiny bit of pancreas made the difference between diabetes and its absence.

Dog C3-86.—Female; mongrel; brown; age 3 years; good condition; weight 15 kilos. Apr. 28, 1916. Removal of pancreatic tissue weighing 33 gm. Remnant about main duct estimated at 2.9 gm. ($\frac{1}{12}$ – $\frac{1}{13}$). Appetite was poor and weight was lost rather rapidly after operation. Glycosuria was absent on bread and soup eaten in small quantities, but present on the addition of 50 gm. of glucose. Sugar freedom on bread diet continued till May 22. Thereafter, 100 gm. of glucose were given by stomach tube before feeding each day. There was glycosuria of 1.8 per cent in 670 cc. of urine on May 23, 0.42 per cent in 312 cc. of urine on May 24, and none thereafter. Failing appetite compelled the stopping of glucose on June 11. Perhaps because of larger eating of bread and soup, glycosuria reappeared on June 15, the body weight being 13.5 kilos.

On that date 0.2 gm. of pancreatic tissue was removed, and at operation it was estimated that no hypertrophy of the remnant had occurred. The specimen was normal microscopically except for vacuolation in a very few cells of the abundant islands. Subsequently the dog proved vigorous up to death on Dec. 12, 1917. The pancreas remnant was soft and appeared normal, and its low weight of 1.6 gm. was perhaps partly due to the fact that emaciation had reduced the body weight to 6.4 kilos.

The apparently high tolerance in this dog was due to poor eating and loss of weight. For the best diabetic experiments it is essential that the dogs should have good appetite and health in other respects.

Dog C3-98.—Male; mongrel; brindle; age 2 years; moderately well nourished; weight 13.4 kilos. June 8, 1916. Removal of pancreatic tissue weighing 25.8 gm. Remnant about main duct estimated at 2.1 gm. ($\frac{1}{13}$). Diabetes was controlled by diet, but its existence was demonstrated by the fact that during 2 months glycosuria could be produced at any time by feeding 100 gm. of bread.

The tolerance was spared by diet until Nov., when the body weight ranged slightly above and below 12 kilos. Bread and soup diet then failed to bring on glycosuria, and during the month Nov. 21-Dec. 21 the daily addition of 200 or 300 gm. of glucose also failed.

Dec. 21. Additional pancreatic tissue weighing 0.85 gm. was removed, the body weight being 11.25 kilos. The dog was unwell thereafter, with diarrhea and poor appetite, so that by Jan. 5, 1917, the body weight had fallen to 9.5 kilos. Glucose feeding as high as 300 gm. daily again failed to produce more than transitory glycosuria, though the dog began to regain weight. Feb. 7. Weight 11.4 kilos; the plasma sugar at 11 a.m. was 0.112 per cent. The usual bread and soup mixture with 300 gm. of glucose was then fed; glycosuria was absent, and at 4 p.m. the plasma sugar was 0.111 per cent.

Feb. 8. 0.5 gm. of additional pancreatic tissue was removed. Diabetes existed thereafter, so that glycosuria could be produced by plain bread and soup diet. June 4. Died; the dog was emaciated down to 8.6 kilos, and the pancreas remnant weighed 2.95 gm.

In the tissue removed Dec. 21, vacuolation of island cells was rare if present, and so slight as to be doubtful. Also on Feb. 8, no positive changes in the islands were demonstrated. The emaciation preceding death was due to other causes than diabetes, and there was no vacuolation of islands.

The long interval of freedom from glycosuria following the first operation permitted actual recovery from the diabetes. This was associated with hypertrophy of the pancreas remnant, and the new formed tissue seemed to be functionally equivalent to the old, for further resection was necessary practically to the same extent as in an original operation, even for an animal at the lower body weight. As usual, carbohydrate overfeeding did not cause degenerative changes in the islands of the non-diabetic animal, and the potential diabetes subsequently in absence of active symptoms was also attended with no island changes.

Glucose Intoxication.

Glucose overfeeding ordinarily causes no harm in dogs beyond diarrhea and loss of weight. In exceptional instances the attempt to produce diabetes has ended in death apparently from poisoning by the prolonged excess of sugar. Gastrointestinal disturbances have appeared sometimes as a prominent cause, and sometimes have been absent or trivial. Though these cases in such a resistant species as dogs are not positive, the genuineness of glucose intoxication is confirmed by fatalities in feeblar animals, such as rabbits and monkeys, and in dogs weakened by a preceding Bernard puncture of the medulla.

Dog B2-48.—Female; mongrel; yellow and white; age 4 years; good condition; weight 14.75 kilos. Mar. 19, 1914. Removal of pancreatic tissue weighing 22.5 gm. Remnant about main duct estimated at 4.7 gm. ($\frac{1}{8}$). Glycosuria being absent on bread and soup diet, on Mar. 30, 200 gm. of glucose were added; the amount was increased on Apr. 1 to 300 gm. As the dog did not like this mixture, on Apr. 9 a mixture of chopped meat, bone-meal, and glucose was substituted, the latter in dosage of 200 or oftener 300 gm. daily. The dog held weight, without glycosuria, and seemed to be thriving until she was unexpectedly found dead on May 9.

Autopsy.—No cause of death was found and no organ changes beyond intense congestion of the liver. Though the animal had been dead several hours, 100 gm. of liver were taken in boiling potassium hydroxide, but the concentrated extract was negative for glycogen. Microscopically, the pancreas remnant (weight 6.8 gm.) and its islands were normal; the liver was strictly normal, except for intense congestion, and was not fatty; the other organs were negative. Death was due to some unknown-intoxication.

Dog B2-69.—Male; bulldog; mongrel; white with black head; age 4 years; good condition; weight 19.9 kilos. May 29, 1914. Removal of pancreatic tissue weighing 36.3 gm. Remnant about main duct estimated at 9.8 gm. ($\frac{1}{4}$ – $\frac{1}{2}$). The dog thrived on bread and soup diet. July 8, 100 gm. of glucose were added, increased on July 15 to 200 gm., on July 20 to 300 gm., and on July 30 to 400 gm., the mixture being improved by the addition of a little chopped meat daily. The weight gradually diminished, but the dog remained lively. Oct. 20. Weight 14 kilos; there was weakness out of all proportion to the emaciation. The animal, though retaining appetite and spirits, could not stand. Glucose was discontinued and meat fed. Oct. 22. The dog was still unable to stand, though wagging his tail and making efforts to rise. On this day was noticed bloody diarrhea, which previously had either been absent or too slight to attract attention. Oct. 23. The animal was found dead.

Autopsy.—The pancreas remnant appeared normal and weighed 16.1 gm. The stomach and intestines appeared normal throughout except for a portion 3 feet in length beginning 6 feet from the pylorus, where the mucous membrane was deeply injected and ecchymotic. The liver and other viscera appeared normal without congestion. There was no noticeable excess or diminution of fluid in the brain or cord. Microscopically the liver, adrenals, and pancreas were found normal. As in Dog B2-48, no fat vacuoles were visible in routine stains of the liver.

Dog B2-49, subjected to similar prolonged glucose feeding, died of gas bacillus infection, which is rare in dogs. Experiments with infection of diabetic dogs with this organism will be published later, but were on the whole negative.

Comparisons of Carbohydrates.

Comparison of Starch and Glucose.—Numerous observations have made it evident that glucose brings on glycosuria and diabetes more actively than starch. The difference is not merely one of quantity, for the addition of 50 or 100 gm. of glucose is sometimes effective when no amount of bread feeding avails for glycosuria. The reason for the readier glycosuria from glucose lies evidently in its quicker absorption, and if distributed in sufficiently small doses throughout the day it would presumably be assimilated as well as starch, as Klemperer showed in human patients. The most important point here is that the sudden glucose flood with its attendant glycosuria is more injurious to the pancreatic function than the more gradual and prolonged labor imposed by starch. It may thus be inferred that sugar is a more dangerous food for human beings with any predisposition to diabetes than is starch.

The question may be raised whether the difference is not merely one of time, and whether starch will not bring on diabetes more slowly than glucose but just as surely. As the difference between the two is not very great, experiments need to be performed with full precautions that the body weight does not vary, that the preliminary tests of the glucose tolerance do not lower the tolerance for starch, and under other conditions of exactness. Examples have already been given in which bread feeding was tolerated for several days before onset of glycosuria. The feature which renders longer tests peculiarly difficult is the recuperative power of the pancreas, which through regeneration reduces the diabetic tendency sometimes to the vanishing point, as described in preceding protocols. This experimental difficulty strengthens the view of the greater danger of sugar for predisposed human individuals, because such power of regeneration as their pancreas may possess will evidently have a better opportunity on starch diet. The difficulty may by extreme care be overcome in very prolonged experiments, in which the animals are kept potentially diabetic for so long a time that the regenerative tendency largely disappears. These conditions were fairly well fulfilled in Dog B2-02, described above. This animal could have been made actively diabetic at any time by glucose except for her repugnance to it. The

feature of incompleteness in the experiment is that death occurred from rabies before diabetes actually ensued on starch diet, but the hydropic degeneration in the islands of Langerhans demonstrated sufficiently that loss of tolerance was in progress and diabetes inevitable.

The record of Dog B2-01 from Sept. to Dec., 1916, to be published later, also shows a downward progress on bread diet, though the diet at first was apparently assimilated even with the addition of 300 gm. of glucose. The same result is shown in the experiments with Dogs D4-52 and D4-69 in Paper 3 of this series.⁶ In the former animal between Aug. 8 and 21, 1917, it was impossible to maintain glycosuria on bread diet with 200 gm. of glucose, and the sugar feeding had to be stopped because of the distaste acquired by the dog for it. The tests of blood and urine on Aug. 8 and 15 also indicated a rise of tolerance during this period. But with continuance of plain bread feeding, glycosuria appeared on Oct. 10, and in a test with the addition of 200 gm. of glucose to the usual bread mixture the excretion was as high as 7 per cent. Likewise Dog D4-69 displayed an apparently increasing assimilation, so that the animal finally tired of glucose and the attempt to maintain glycosuria apparently failed. But with continuance of bread feeding, glycosuria began on Oct. 6, and, just as with Dogs B2-01 and D4-52, the tolerance proved to be permanently lowered thereafter.

When the regenerative power of the pancreas is not so great as to result in a practical cure of the diabetes, the difference between starch and glucose seems to be essentially one of time and degree. Glucose through its quicker absorption floods the body more suddenly and violently, but starch works the same damage more slowly but just as surely. This was shown in several animals through the onset of diabetes, and in one by examination of the islands of Langerhans. By inference, if human diabetes is so mild that glycosuria ceases on withdrawal of sugar without limitation of other carbohydrate, it is not correct to treat the case in this manner or to leave either starch or total calories unrestricted.

When the regenerative power of the pancreas is sufficient, there is a real difference due to the time element mentioned, and a number of examples have already been given of more or less complete recovery from diabetes on starch diet by dogs which could readily have been made diabetic by forcing of glucose shortly after operation. Some-

⁶ Allen, F. M., *J. Exp. Med.*, 1920, xxxi (in press).

thing similar may conceivably occur in some human patients. In properly chosen animals without too strong a tendency either to diabetes or to recovery, it is possible to demonstrate a recuperative attempt by functional tests showing an apparent increase of assimilation, but the continued overtaxing of the assimilation causes its breakdown, and recovery to the former extent is never again possible. A similar gain of tolerance is well known in human patients under dietary restriction in the earlier stages of diabetes, and the warning against abusing this recuperative tendency is similar, especially as actual recovery on the part of human patients has seldom been witnessed.

Three of the four dogs here mentioned were kept sufficiently long to demonstrate that the lowering of tolerance referred to was actually the result of the carbohydrate excess and not of any inherent tendency in the dogs. The onset of diabetes was evidently checked by change to protein-fat diets, and especially in Dog B2-01 the tolerance could be manipulated up and down by changing the body weight. It is therefore confirmed that carbohydrate injures the assimilation more rapidly and powerfully than any other food. The later observations show also the harmfulness of *luxus* diets, but these diets are most dangerous when they include carbohydrate.

Comparisons of Starches.

Experiments were begun to test the glycosuric effect of different starchy foods, in relation to the hypotheses of certain writers concerning carbohydrate "cures." Dogs with potential diabetes of long standing and with fairly stationary assimilative power were given quantities of carbohydrate corresponding to the limits of their known tolerance. Stale bread was used, with a carbohydrate content roughly similar to that of the cereals. The bread was moistened with water after weighing. The cereals were weighed raw, and then boiled. No other food was given on the test days, and a fixed diet of 1 kilo of beef lung was followed on all other days. There was never glycosuria except on test days.

On test days approximately 50 gm. of starch were administered to Dog B2-43 in the following forms:

Dog B2-43.

Date.	Food.	Glycosuria.
<i>1914</i>		<i>gm.</i>
Sept. 10	Oatmeal.	0
" 15	Rice.	5.5
Oct. 2	Potato.	0
" 10	Bread.	6.4
" 19	Pearled barley.	2.3

The program was continued in the same animal by varying the quantities of the different foods, with a view to testing the tolerance for each.

Date.	Diet.	Glycosuria.
<i>1915</i>		<i>gm.</i>
Mar. 26	75 gm. oatmeal.	0
" 30	100 " "	0
Apr. 1	200 " "	0.7
" 16	50 " bread, 1 kilo lung.	Faint.
" 19	50 " " 1 " "	0
" 20	100 " " 1 " "	Slight.
" 24	100 " "	3.0
" 27	100 " oatmeal.	0
May 3	100 " "	0
" 5	150 " "	0
" 10	200 " "	Faint.
" 17	200 " " 1 kilo lung.	0
" 25	200 " rice.	8.4
June 1	100 " "	6.4
" 8	50 " "	Faint.
" 15	200 " oatmeal.	3.4
" 26	200 " rice.	1.6

Dog B2-63.

Date.	Diet.	Glycosuria.
<i>1915</i>		<i>gm.</i>
Apr. 16	50 gm. bread.	0
" 20-23	100 " " 1 kilo lung daily.	Traces.
" 24	150 " " 1 " "	1.6
" 27	150 " oatmeal.	0
May 3	150 " "	2.6
" 10	200 " "	2.3
" 17	200 " " 1 kilo lung.	1.1
" 25	200 " rice.	5.6
June 1	100 " "	11.7
" 8	50 " "	Trace.
" 15	200 " oatmeal.	"
" 25	200 " rice.	0
July 3	200 " bread.	0
" 6	200 " "	0

Dog B2-71.

Date.	Diet.	Glycosuria.
<i>1915</i>		<i>gm.</i>
Mar. 29	100 gm. bread.	0
" 30	200 " "	11.4
" 31	Fast day.	0
Apr. 1	300 gm. bread.	6.8
" 16	50 " " 1 kilo lung.	0
" 20	100 " " 1 " "	0
" 24	100 " "	1.4
" 27	100 " oatmeal.	0
May 3	100 " "	Trace.
" 5	150 " "	0
" 10	200 " "	8.7
" 17	200 " " 1 kilo lung.	9.1
" 25	200 " rice.	19.3
June 1	100 " "	18.7
" 8	50 " "	0
" 15	200 " oatmeal.	0
" 26	200 " rice.	2.2

Dog B2-76.

On test days approximately 50 gm. of starch were administered as follows:

Date.	Food.	Glycosuria.
<i>1914</i>		<i>gm.</i>
Sept. 10	Oatmeal.	Trace.
" 15	Rice.	3.1
Oct. 2	Potato.	1.1
" 10	Bread.	2.8
" 19	Pearled barley.	1.2

Dog B2-81.

Date.	Diet.	Glycosuria.
<i>1915</i>		<i>gm.</i>
Mar. 31	200 gm. bread.	0
Apr. 1	300 " "	0.73
" 16	50 " "	0
" 19	50 " " 1 kilo lung.	0
" 20	100 " " 1 " "	2.8
" 24	100 " "	0.16
" 27	100 " oatmeal.	0
May 3	100 " "	0
" 5	100 " "	0
" 10	200 " "	0
" 17	200 " " 1 kilo lung.	3.8
" 25	200 " rice.	5.3
June 1	100 " "	7.4
" 8	50 " "	0
" 15	200 " oatmeal.	Trace.

The claims for the superior assimilability of certain starchy foods, particularly oatmeal, have been previously reviewed.⁷ As the claims included some observations with totally depancreatized dogs, the above tests were begun upon partially depancreatized animals, which more closely resemble human diabetics in digestive power and other respects. They were intended as orientation experiments, to decide whether an investigation with combined analyses of the food, feces, and blood sugar was worth while. The following deductions were made.

Precautions were used against fluctuations of assimilation due to changes of body weight, indigestion, diet in the intervals between test days, and other known factors. Dog B2-63 obviously gained tolerance toward the close of the experimental period; otherwise there was success in the attempt to choose animals with fairly stationary tolerance. In Dog B2-71, a single fast day on March 31 evidently increased the bread tolerance considerably, as shown by comparison of March 30 and April 1. With all care against disturbing influences, the tolerance of the animals shows occasional fluctuations from unknown causes, which would seriously hamper accurate experiments upon such test objects. The milder cases of human diabetes, which have been used for comparisons of carbohydrate tolerance, are subject to fully as great fluctuations of tolerance as these animals, and the exactness of tests upon them is open to similar question.

Owing to such accidental fluctuations, the assimilation of oatmeal might rarely seem inferior to that of rice, as in Dog B2-43 on June 15 and 26. But the number of observations was great enough to establish the rule that glycosuria from oatmeal was in general much less than that from any other carbohydrate. Generally the dogs ate the oatmeal less willingly than other foods, notably bread and rice, which caused greater glycosuria. Even when it was eaten promptly, and in the absence of diarrhea, there is the usual question whether the absorption is actually equal, and the recognized impossibility of de-

⁷ Allen,¹ Chapter IX. Allen, F. M., Stillman, E., and Fitz, R., Total dietary regulation in the treatment of diabetes, Monograph of The Rockefeller Institute for Medical Research, No. 11, New York, 1919, Chapter I.

termining the rate of absorption or the extent of fermentation by fecal analyses or any other means. The above findings in dogs are similar to the clinical observations on which the oatmeal treatment was based, but the calorimetric studies of Du Bois⁸ confirmed the more modern view that the assimilation of oatmeal is not actually superior to that of other starchy foods. When this more accurate method became available, the animal experiments were dropped.

Carbohydrate fed at the same time with protein in the form of beef lung sometimes caused slightly greater glycosuria than the carbohydrate alone. For example, Dog B2-71 showed slight differences of this character with oatmeal on May 10 and 17, and Dog B2-81 with bread on April 20 and 24, and with oatmeal on May 17 and June 15. But in equally numerous instances it is seen that the glycosuria was greater from carbohydrate alone (Dog B2-43, April 20 and 24, May 10 and 17; Dog B2-63, May 10 and 17; Dog B2-71, April 20 and 24), as if the mixture with protein had slowed the absorption of the carbohydrate. The observations are entirely contrary to the claim that the assimilability of oatmeal is spoiled by simultaneous ingestion of meat.

CONCLUSIONS.

1. The injurious effects of excessive carbohydrate diet are demonstrable in partially depancreatized dogs, in the same manner as in human patients. With severe diabetes there is rapid progress of emaciation and weakness and early death.

2. With milder diabetes, there is frequently a transitional state following operation, when the fate depends on the diet. If the tolerance is spared for a time, recovery sometimes occurs to such extent that diabetes cannot be produced by any kind or quantity of feeding, but only by removal of a small additional fragment of pancreatic tissue. The proper degree of carbohydrate overfeeding is important in this early period for producing the most useful type of diabetic animals; namely, those having good digestion and general health combined with a permanent lowering of assimilative power, like the condition of human patients.

⁸ Allen, F. M., and Du Bois, E. F., *Arch. Int. Med.*, 1916, xvii, 1010.

3. In the early stage, glucose is more powerful than starch in producing diabetes, and animals which are progressing toward complete recovery on starch diet can be sent into hopeless diabetes by admixture of glucose. The difference seems to be merely of the rate of absorption, and indicates that a rapid flood of carbohydrate is more injurious to the pancreatic function than a slow absorption. Whenever permanent diabetes is present, so that complete recovery is impossible, starch brings on glycosuria more slowly than sugar, but just as surely. The difference in time in different cases amounts to days, weeks, or months. The clinical lesson from such experiments is that even if a patient becomes free from glycosuria on withdrawal of sugar only, nevertheless other foods should also be limited.

4. No significant differences were observed between the assimilation of different starches, or any extreme lowering of the carbohydrate tolerance by proteins, such as alleged by certain writers in connection with the "oatmeal cure."

5. Repair of traumatic inflammation and hypertrophy of the pancreas remnant have been mentioned incidentally as the basis of the early tendency to recovery, and also hydropic degeneration of Langerhans islands as an accompaniment of the lowering of tolerance by excessive diet. These are believed to have their parallels in human cases, and are to be described more fully hereafter.

STUDIES ON EXPERIMENTAL PNEUMONIA.*

I. PRODUCTION OF PNEUMOCOCCUS LOBAR PNEUMONIA IN MONKEYS.

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PLATE 21.

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INTRODUCTION.

The unusual prevalence of pneumonia during the past 2 years has led to much intensive study of the disease. Perhaps the most important contributions to these studies have been the increasing emphasis laid upon etiologic classification of pneumonia, the correlation of the clinical features and pathology of the disease with the various bacteria that are capable of producing it, and the indication that prophylactic vaccination against certain types of pneumococcus pneumonia may lead to a reduction in the incidence of these types.

The different biologic types of pneumococcus, *Streptococcus hæmolyticus*, and *Bacillus influenzae* have been the organisms most frequently associated with the pneumonia that has occurred. While it has been found possible to differentiate to a considerable extent the clinical features and pathology of the various types of pneumonia caused by these different bacteria, many points have remained obscure because of the frequent occurrence of mixed infections, especially in the pneumonia that has followed influenza. Perhaps the greatest difference in opinion has arisen with respect to the relation of *Bacillus influenzae* to epidemic influenza and to the pneumonia which so frequently complicates it. Similarly, though the results of prophylactic vaccina-

* The authors desire to express their indebtedness to Colonel F. F. Russell, Medical Corps, U. S. Army, and to Lieutenant Colonel H. J. Nichols, Medical Corps, U. S. Army, through whose cooperation every facility for the conduct of these experiments was made available; also to Second Lieutenant Gustav I. Steffen, Sanitary Corps, U. S. Army, for technical assistance throughout the course of the investigation.

tion against pneumococcus pneumonia strongly suggest that a definite degree of immunity against lobar pneumonia is provided by this procedure, the interpretation of statistical studies upon the incidence of disease in man is always difficult because of uncontrollable and apparently unrelated factors that may influence the outcome of the experiment.

It seemed possible that more definite knowledge might be obtained concerning the various types of pneumonia and the efficiency of prophylactic vaccination if a study of experimental pneumonia were undertaken, since by this means pure infections might be dealt with and adequate experimental controls would be available. In addition, a study of the therapeutic efficiency of Type I antipneumococcus serum and of an outbreak of spontaneous pneumonia among the stock animals was conducted.

Since it had been previously found¹ that experimental pneumonia could readily be produced in the monkey by the intratracheal injection of small amounts of culture, this animal was selected as offering the best opportunity for success. Two species of monkeys have been used, *Macacus syrichtus* from the Philippine Islands and *Cebus capucinus* from Central America. All were fresh stock and had not previously been kept in captivity in this country. Normal animals were used throughout, no preliminary procedures to lower resistance or to produce injury to the respiratory tract being resorted to except in a few instances for special purposes as indicated in the text. The results of the study will be reported in a series of papers.²

¹ Opie, E. L., Freeman, A. W., Blake, F. G., Small, J. C., and Rivers, T. M., unpublished experiments.

² The present paper is the first of this series; Paper II follows. The others will appear in later numbers of this *Journal*. Their titles are as follows: III. Spontaneous pneumonia in monkeys. IV. Results of prophylactic vaccination against pneumococcus pneumonia in monkeys. V. Active immunity against experimental pneumococcus pneumonia in monkeys following vaccination with living cultures of pneumococcus. VI. Active immunity following experimental pneumococcus pneumonia in monkeys. VII. Treatment of experimental Pneumococcus Type I pneumonia in monkeys with Type I antipneumococcus serum. VIII. Experimental *Streptococcus hemolyticus* pneumonia in monkeys. IX. Production of influenza in monkeys by inoculation with *Bacillus influenzae*. X. Pathology of experimental influenza and of *Bacillus influenzae* pneumonia in monkeys.

Pneumococcus Lobar Pneumonia.

Many attempts have been made to produce lobar pneumonia in animals.³ In the instances in which a lobar consolidation of the lung has been successfully produced, the results have not been altogether satisfactory either because it has not been clearly shown that the animals suffered from clinical lobar pneumonia as seen in man, or because large amounts of culture material were injected under more or less artificial conditions, or because various preliminary procedures injurious to the respiratory tract were resorted to in order to render the animal susceptible to infection. In the present experiments the attempt has been made to avoid these objections in as far as was possible, while at the same time a method of inoculation was employed that would consistently produce the desired result.

Four highly virulent strains of pneumococcus have been used throughout. Three of these were stock laboratory strains originally isolated from cases of lobar pneumonia of *Pneumococcus* Type I, *Pneumococcus* Type II, and *Pneumococcus* Type III respectively, of which 0.0000001 cc. of an 18 hour broth culture injected intraperitoneally regularly killed white mice within 48 hours. The fourth was a pneumococcus of Type IV isolated at autopsy from a monkey dying of spontaneous lobar pneumonia. This pneumococcus killed white mice within 48 hours when injected intraperitoneally in doses of 0.000001 cc. of an 18 hour broth culture.

Methods.

The method of inoculation was by direct intratracheal injection, under aseptic precautions, by the insertion of a small caliber, dry, sterile needle into the lumen of the trachea between the tracheal cartilages just below the larynx and the introduction of the culture by means of a Luer glass syringe inserted into the stock of the needle.

³ Wadsworth, A., *Am. J. Med. Sc.*, 1904, cxxvii, 851. (This article contains a review of the older literature.) Lamar, R. V., and Meltzer, S. J., *Proc. Soc. Exp. Biol. and Med.*, 1909-10, vii, 102; *J. Exp. Med.*, 1912, xv, 133. Wollstein, M., and Meltzer, S. J., *J. Exp. Med.*, 1912, xvi, 126. Winternitz, M. C., and Hirschfelder, A. D., *J. Exp. Med.*, 1913, xvii, 657. Sisson, W. R., and Walker, I. C., *J. Exp. Med.*, 1915, xxii, 747.

The procedure is relatively simple, takes but a few seconds, subjects the animal to a minimal amount of trauma, and is not attended by gagging or coughing. In no instance at autopsy has there been any evidence of local injury or infection in the trachea or in the tissue surrounding it. The amounts of culture injected ranged from 0.000001 to 1 cc. of an 18 hour plain broth culture. A constant amount of fluid, 1 cc., was used throughout, dilutions of cultures being made in plain broth.

The methods of study after inoculation consisted in observation and record of clinical symptoms and physical signs, the taking of morning and afternoon temperature (rectal), daily counts of the white blood corpuscles with examination of stained blood films and differential white blood counts, and daily blood cultures in broth and in poured agar plates with record of the number of pneumococcus colonies developing per 0.5 cc. of blood. No record of the pulse or respiration rate was kept, since it was not found feasible to record these accurately. X-ray plates were taken in a few instances (Figs. 1 and 2), but no systematic study was made. Autopsies with bacteriological examination were performed on all animals that died or were killed.

EXPERIMENTAL.

Production of Lobar Pneumonia by the Intratracheal Injection of Pneumococcus.

Thirty-seven normal monkeys were injected intratracheally with cultures of pneumococcus in amounts ranging from 1 to 0.000001 cc. of an 18 hour broth culture, a constant amount of fluid, 1 cc., being used in all the experiments. Pneumococcus Type I was used thirty-one times; Pneumococcus Type II, twice; Pneumococcus Type III, three times; and Pneumococcus Type IV, once. In thirty-two instances lobar pneumonia in all its aspects resembling the disease as seen in man was successfully produced. In five cases the monkeys failed to develop pneumonia. The results are shown in Table I.

It will be seen from the data presented in Table I that pneumonia was as readily produced by the injection of 0.000001 cc. of pneumococcus as with larger amounts. Of the twenty-six monkeys in which

lobar pneumonia was produced with *Pneumococcus* Type I, twenty-one died, and five recovered, the latter all receiving amounts of less than 0.001 cc. With the exception of the fact that all the monkeys which received 0.001 cc. of culture or more died, there is no evident relation between the duration of the disease and final outcome on the one hand and the weight of the monkey or amount of culture injected on the other. The differences probably depended upon individual variation in resistance to pneumococcus infection.

Although the strains of *Pneumococcus* Type II, Type III, and Type IV used were as virulent for mice as the strain of *Pneumococcus* Type I, they proved much less so for monkeys. Of the six monkeys injected with these organisms all recovered, although a relatively large amount of culture, 0.1 to 1 cc., was used.

No attempt is made to explain the failure to produce pneumonia in five animals. Monkey 4 died in 23½ hours with an overwhelming septicemia. Monkey 27 died on the 3rd day with an acute fibrinopurulent pneumococcus pericarditis and overwhelming septicemia. Monkeys 63, 76, and 128, all of which received minute amounts of culture, showed no evidence of infection.

In addition to the cases shown in Table I, lobar pneumonia was successfully produced in a similar manner in twenty-nine other experiments, twenty times with *Pneumococcus* Type I, twice with *Pneumococcus* Type II, twice with *Pneumococcus* Type III, and five times with *Pneumococcus* Type IV. These monkeys had either been vaccinated previously with pneumococcus vaccine, or had had a preceding attack of pneumonia, or were treated with antipneumococcus serum, and therefore, although they were in normal health at the time of injection, they are excluded from the series of normal animals shown in Table I. Experiments dealing with them will be presented in Papers IV to VII. The clinical records of some, however, are included in this paper in order to illustrate certain features of the disease.

In the following paragraphs abbreviated protocols illustrative of the clinical features of the pneumonia experimentally produced in monkeys are presented, study of the pathology of the disease being reserved for Paper II. Since many of the experiments were entirely similar, it has not seemed necessary to give the protocols of all, those

TABLE I.
Effect of Intratracheal Inoculation of Monkeys with Pneumococcus.

Monkey No.	Species.	Weight. gm.	Date of inoculation. 1919.	Type of pneumococcus.	Amount of culture.	Clinical diagnosis.	Result.	Autopsy.
1	<i>C. capricinus</i> .	1,716	Feb. 17	I	cc. 1.0	L. P.*	Died on 2nd day.	Lobar pneumonia, R. L.,† engorgement and red hepatization.
2	"	1,290	" 24	I	0.5	"	" in 12½ hrs.	Lobar pneumonia, R. U., R. L., L. U., L. L.; engorgement and red hepatization.
3	"	1,595	" 24	I	0.1	"	" on 2nd day.	Lobar pneumonia, L. U., L. L.; engorgement and red hepatization.
4	"	1,835	" 26	I	0.01	?	" in 23½ hrs.	No pneumonia. Pneumococcus septicaemia.
5	"	1,410	Mar. 5	I	0.01	L. P.	" " 23¼ hrs.	Lobar pneumonia, R. U., R. L., L. L.; engorgement and red hepatization.
6	"	1,960	" 5	I	0.001	"	" on 4th day.	Lobar pneumonia, R. U., R. L.; engorgement and red hepatization; acute fibrinous pleuritis, right.
23	<i>M. syriacus</i> .	3,555	" 18	I	1.0	"	" " 5th "	Lobar pneumonia, L. L., engorgement and red hepatization; R. L., engorgement; acute fibrinous pleuritis, left.
26	"	2,680	" 21	I	1.0	"	Dying, killed 9th day.	Lobar pneumonia, R. L., L. L.; gray hepatization; acute fibrinous pleuritis, right and left.
27	"	2,620	" 26	I	1.0	P. S.	Died on 3rd day.	Acute fibrinopurulent pericarditis; pneumococcus septicaemia; no pneumonia.
29	"	3,920	Apr. 2	I	1.0	L. P.	" " 2nd "	Lobar pneumonia, L. U., L. M., L. L.; engorgement and red hepatization; acute fibrinous pleuritis, left.

30	<i>M. syrichtus</i> .	4,120	Apr.	2	I	0.1	L. P.	Died on 12th day.	Lobar pneumonia, R. U., R. M., R. L., L. U., L. M., L. L.; gray hepatization and beginning resolution; acute fibrinous pleuritis, right and left; dilatation of heart.
31	"	3,850	"	2	I	0.01	"	" " 10th "	Lobar pneumonia, L. U., L. M., L. L.; gray hepatization; empyema, left; acute purulent mediastinitis.
25	<i>C. capucinus</i> .	740	"	4	II	0.1	"	Recovered by crisis on 7th day. Killed.	Lobar pneumonia, R. U.; gray hepatization and beginning resolution; acute fibrinous pleuritis, right.
42	<i>M. syrichtus</i> .	4,955	"	10	I	0.001	"	Died on 37th day.	Lobar pneumonia, L. M., L. L., organization; R. U., R. M., R. L., partial resolution and organization; chronic fibrinous pleuritis, right and left.
63	"	2,100	"	19	I	0.000001	†	Remained well.	No autopsy.
75	"	4,200	"	29	I	0.0001	L. P.	Recovered by crisis on 12th day.	" "
76	"	4,600	"	29	I	0.00001	†	Remained well.	" "
77	"	2,600	"	29	I	0.000001	L. P.	Recovered by crisis on 11th day.	" "
82	<i>C. capucinus</i> .	730	"	30	III	0.1	"	Recovered by crisis on 7th day. Killed.	Interstitial pneumonia, R. U.; resolving.
83	<i>M. syrichtus</i> .	2,470	"	30	I	0.001	"	Died on 8th day.	Lobar pneumonia, R. U., R. M., R. L.; red and gray hepatization; acute serofibrinous pleuritis, right.
85	"	2,675	May 6	I	I	0.001	"	" " 6th "	Lobar pneumonia, L. M., L. L.; engorgement and red hepatization; acute fibrinous pleuritis, left.

* L. P indicates lobar pneumonia; P. S., pneumococcus septicemia.

† R. L., R. M., R. U., etc., indicate lobes of the lung. The cardiac lobe is included as part of the right lower lobe.

‡ No disease.

TABLE I—*Concluded.*

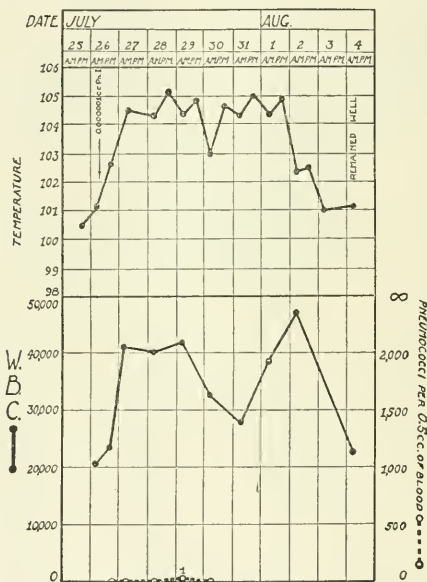
Monkey No.	Species.	Weight. <i>gm.</i>	Date of inoculation.	Type of pneumococcus.	Amount of culture.	Clinical diagnosis.	Result.	Autopsy.
86	<i>M. syriditus.</i>	2,424	1919 May 6	I	cc. 0.000001	L. P.	Died on 14th day.	Lobar pneumonia, R. L., L. L.; gray hepatization; acute fibrinous pleuritis, right and left.
87	"	2,835	" 6	I	0.1	"	" " 5th "	Lobar pneumonia, L. U., L. M., L. L., red hepatization; R. L., engorgement; acute fibrinous pleuritis, left.
95	"	4,100	" 13	I	0.00001	"	Recovered by crisis on 11th day. Relapse (?) on 15th day. Recovery by crisis on 21st day. Killed.	Lobar pneumonia, L. L.; partial resolution and organization; organizing pleuritis, left.
96	"	2,655	" 13	I	0.000001	"	Recovered by crisis on 9th day.	No autopsy.
93	"	5,110	" 15	I	0.001	"	Died on 13th day.	Lobar pneumonia, R. U., R. M., R. L.; gray hepatization; acute fibrinous pleuritis, right; hypertrophy and dilatation of heart.
98	"	4,100	" 20	I	0.000001	"	" " 4th "	Lobar pneumonia, R. U., R. L., L. U., L. L.; engorgement.
109	"	4,585	" 27	I	0.001	"	" " 3rd "	Lobar pneumonia, R. U., R. M., L. U., L. M.; engorgement.
110	"	2,710	" 27	I	0.000001	"	" " 7th "	Lobar pneumonia, R. U., R. M., R. L.; red and gray hepatization; acute fibrinopurulent pericarditis; acute fibrinous pleuritis, right.

111	<i>M. syrichtus</i> .	2,220	June 27	IV	0.1	L. P.	Recovered by crisis on 11th day.	No autopsy.
112	"	3,975	" 5	I	0.01	"	Died on 6th day.	Lobar pneumonia, R. M., R. L., red and gray hepatization; L. L., engorgement; acute fibrinous pleuritis, right.
114	"	3,000	" 20	I	0.000001	"	" " 5th "	Lobar pneumonia, R. L., L. L.; engorgement.
91	"	4,005	" 24	II	0.1	"	Recovered by crisis on 18th day.	No autopsy.
115	"	3,015	" 24	III	0.1	"§	Recovered by lysis on 3rd day.	" "
107	"	2,335	July 1	III	1.0	"§	Recovered by lysis on 4th day. Killed.	Interstitial pneumonia, R. L.; acute fibrinous pleuritis right.
127	"	2,700	" 24	I	0.000001	"	Recovered by crisis on 8th day.	No autopsy.
128	"	2,650	" 26	I	0.000001	"†	Remained well.	" "

§ Abortive.

presented having been selected with the purpose of bringing out as fully as possible the various aspects of the disease. The temperature, white blood counts, and results of blood cultures are shown in Text-figs. 1 to 17.

Experiment 1. Monkey 115 (Text-Fig. 1).—Macacus syrichtus, female; weight 3,015 gm. Previously inoculated with Pneumococcus Type III on June 24, and



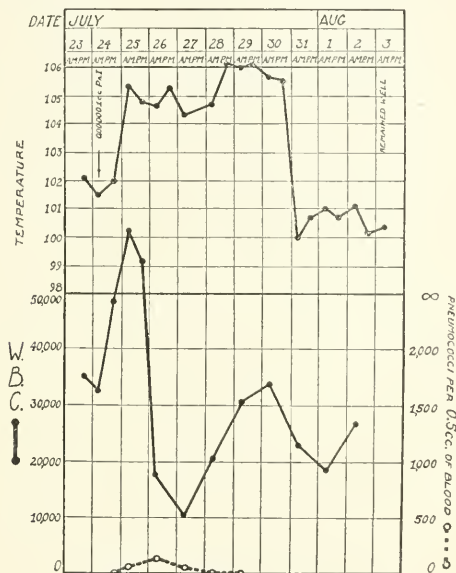
TEXT-FIG. 1. Monkey 115. Experimental lobar pneumonia following the intratracheal injection of 0.000001 cc. of *Pneumococcus* Type I broth culture.

with *Pneumococcus* Type IV on July 1, 1919. July 25. Well and active. July 26, 10.20 a.m. Intratracheal injection of 0.000001 cc. (in 1 cc.) of 18 hour broth culture of *Pneumococcus* Type I. 4 p.m. Appears well. July 27. Moderately sick; respirations rapid and labored, with expiratory grunt. July 28. Condition the same. July 29. Quite sick; no appetite; breathing rapidly; coughs frequently.

Dullness and suppressed breath sounds over right lower lobe. July 30 to Aug. 1. Condition the same. Aug. 2. Appears well and active; breathing easily; temperature fallen to normal by crisis. Aug. 4. Continues well.

Diagnosis.—Lobar pneumonia, right lower lobe.

Experiment 2. Monkey 117 (Text-Fig. 2).—*Macacus syrichthus*, male; weight 2,565 gm. July 23, 1919. Well and active. July 24, 11 a.m. Intratracheal injection of 0.000001 cc. (in 1 cc.) of 18 hour broth culture of *Pneumococcus* Type

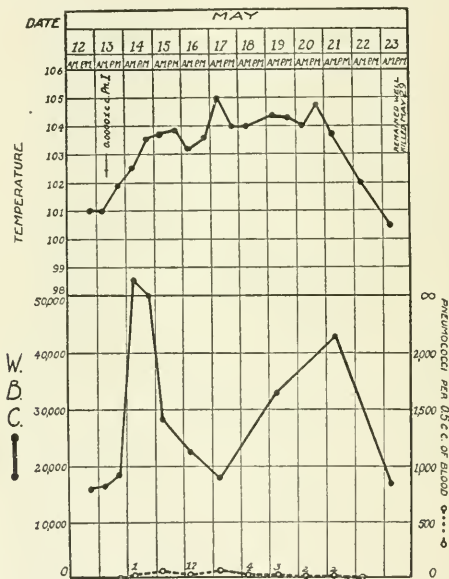


TEXT-FIG. 2. Monkey 117. Experimental lobar pneumonia following the intratracheal injection of 0.000001 cc. of *Pneumococcus* Type I broth culture.

I. 4.30 p.m. Appears well. July 25, 11 a.m. Shivering violently. Respirations accelerated. July 26. Sick; respirations rapid and labored; lungs clear. July 27. Condition the same. July 28. Refuses food; breathing rapidly; dullness over right lower lobe. July 29. Condition the same. Marked dullness, suppressed breathing, and fine moist râles over right lower lobe; coughs occasionally. July 30. Condition the same. July 31. Appears well; breathing easily; temperature fallen to normal by crisis. Aug. 5. Continues well.

Diagnosis.—Lobar pneumonia, right lower lobe.

Experiment 3. Monkey 81 (Text-Fig. 3).—*Macacus syrichtus*, female; weight 2,750 gm. May 12, 1919. Well and active. May 13, 10.55 a.m. Intratracheal injection of 0.00001 cc. (in 1 cc.) of 18 hour broth culture of *Pneumococcus* Type I. 4.20 p.m. Appears well. May 14. Quiet but otherwise appears well. May 15. Moderately sick; breathing rapidly. May 16 to 18. Condition the same. May 19 to 22. Appears sicker; huddled up leaning against side of cage; respira-



TEXT-FIG. 3. Monkey 81. Experimental lobar pneumonia following the intratracheal injection of 0.00001 cc. of *Pneumococcus* Type I broth culture.

tions rapid and labored. May 23. Appears much better, but manifests considerable weakness and dyspnea on exertion; temperature normal. May 25. Appears stronger. May 29. Remains well. Killed.

Autopsy.—Lobar pneumonia, right middle and lower lobes, resolving; acute fibrinous pleuritis, right.

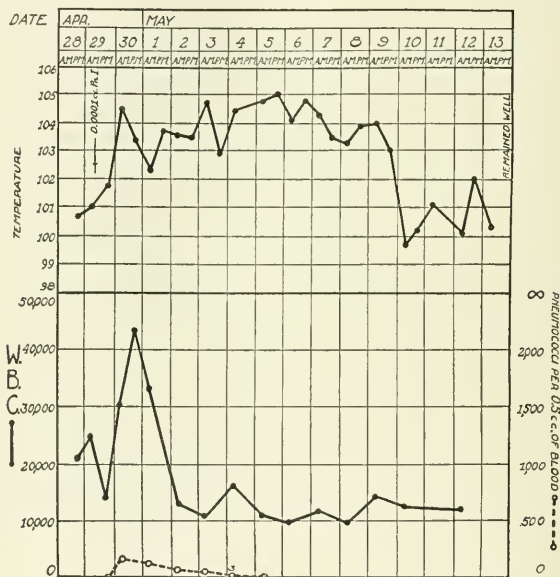
Cultures.—Heart's blood and right lower lobe, no growth.

Monkeys 115, 117, and 81 show well the characteristic clinical features of relatively mild lobar pneumonia with recovery. It will be noted that the temperature curves are identical with those of similar cases in man, presenting the features of sudden onset, sustained elevation throughout the course of the disease, and a critical fall to normal on the 7th or 8th day. The characteristic chill at onset was observed in Monkey 117. In relatively mild pneumonia such as these cases represent monkeys appear only moderately sick, sitting up throughout the course of the disease, and never exhibiting any degree of prostration. The clinical symptoms consist of characteristic rapid and labored respiration, often with expiratory grunt, infrequent cough rarely appearing before the latter half of the disease, and loss of appetite and activity. Monkeys 115 and 117 show a characteristic transient invasion of the blood by pneumococci with disappearance of the bacteremia several days before crisis. Monkey 81 shows a low grade bacteremia throughout the active stage of the disease, an uncommon feature in monkeys that recovered. The characteristic leucocyte curve in non-fatal pneumonia is well illustrated in all three cases. It consists of a preliminary high polymorphonuclear leucocytosis followed by a fall to normal, a secondary rise during the latter half of the disease, and a return to normal with crisis. It will be noted that the first fall of leucocytes to normal is approximately coincident with the peak of the bacteremia, a feature that has occurred with surprising constancy throughout the series of experiments. This type of leucocyte reaction has occurred in all monkeys that have recovered, with the exception of four in which the secondary rise was not demonstrated.

Experiment 4. Monkey 75 (Text-Fig. 4).—Macacus syrichtus, male; weight 4,200 gm. Apr. 28, 1919. Well and active. Apr. 29, 11.10 a.m. Intratracheal injection of 0.0001 cc. (in 1 cc.) of 18 hour broth culture of Pneumococcus Type I. 5.10 p.m. Appears well. Apr. 30, 11 a.m. Sits huddled up, shivering; respirations slightly accelerated. May 1. Sick; refuses food; respirations rapid with expiratory grunt. Moderate dullness, diminished breath sounds, and fine moist râles in left lower axilla. May 2. Condition the same. Dullness and bronchial breathing over left lower lobe. May 3 to 8. Condition remains the same; coughs frequently. May 9. Improving. May 10. Seems well and active, though weak. Temperature fallen to normal by crisis. May 15. Remains well. X-ray photograph of chest shows an area of density in the left lower chest (Fig. 1).

Diagnosis.—Lobar pneumonia, left lower lobe.

Monkey 75 illustrates a prolonged severe case of lobar pneumonia with final recovery by crisis on the 12th day. The sudden onset with chill, the rapid respiration with expiratory grunt, and the physical signs are all characteristic of typical lobar pneumonia. The diagnosis was confirmed by x-ray. The absence of a secondary rise in the



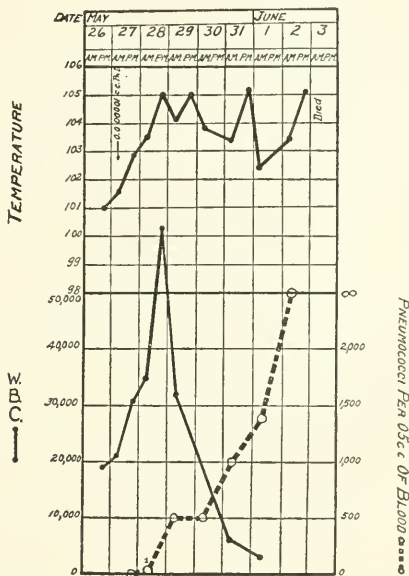
TEXT-FIG. 4. Monkey 75. Experimental lobar pneumonia following the intratracheal injection of 0.0001 cc. of *Pneumococcus* Type I broth culture.

leucocyte curve is illustrated in this case, a feature of unusual occurrence in monkeys that recovered.

*Experiment 5. Monkey 110 (Text-Fig. 5).—Macacus syrichtus, female; weight 2,710 gm. May 26, 1919. Well and active. May 27, 10.20 a.m. Intratracheal injection of 0.000001 cc. (in 1 cc.) of 18 hour broth culture of *Pneumococcus* Type I. May 28. Quiet; respirations rapid. May 29. Appears moderately*

sick; refuses food; breathing rapidly. May 30. Condition the same. May 31 to June 1. Very sick; respirations rapid and labored; sits huddled up in corner of cage. June 2. Very sick; respirations rapid and gasping; lies down on floor of cage at times. June 3. Found dead in morning.

Autopsy.—Lobar pneumonia, right upper, middle, and lower lobes; red and gray hepatization; acute fibrinopurulent pericarditis; acute fibrinous pleuritis, right; cardiac dilatation.

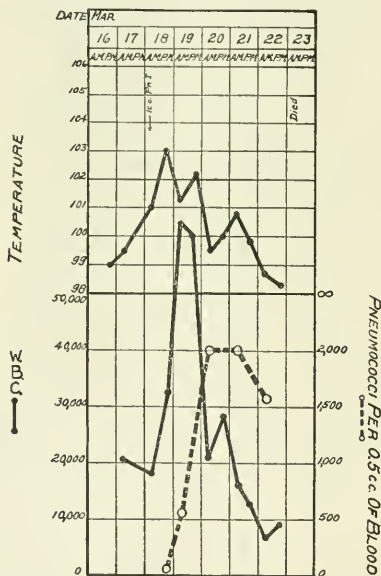


TEXT-FIG. 5. Monkey 110. Experimental lobar pneumonia following the intratracheal injection of 0.000001 cc. of *Pneumococcus* Type I broth culture.

Cultures.—Heart's blood, bronchus, right lower lobe, and pericardial fluid, *Pneumococcus* Type I.

Experiment 6. Monkey 23 (Text-Fig. 6).—*Macacus syrichtus*, male; weight 3,555 gm. Mar. 17, 1919. Well and active. Mar. 18, 10 a.m. Intratracheal injection of 1 cc. of 18 hour broth culture of *Pneumococcus* Type I. Mar. 19, 10 a.m. Appears more quiet than usual, but appetite is good and animal moves about

2 $\frac{3}{4}$ p.m. Appears sick; breathing rapidly; lungs clear. X-ray of chest shows slightly increased density in the left mid-lung area. Mar. 20. Sick; respirations rapid and grunting; refuses food; abdomen distended and tense. Moderate dullness and distant breath sounds in left lower axilla. Mar. 21. Very sick; respirations rapid and labored. Examination of chest shows pleural friction rub, dullness, and faint bronchial breathing in left lower axilla. Mar. 22. Condition the same. Mar. 23. Found dead in morning.



TEXT-FIG. 6. Monkey 23. Experimental lobar pneumonia following the intratracheal injection of 1 cc. of Pneumococcus Type I broth culture.

Autopsy.—Lobar pneumonia, left lower lobe; engorgement and red hepatization; incipient lobar pneumonia, right lower lobe; acute fibrinous pleuritis, left.

Cultures.—Heart's blood and left lower lobe, Pneumococcus Type I.

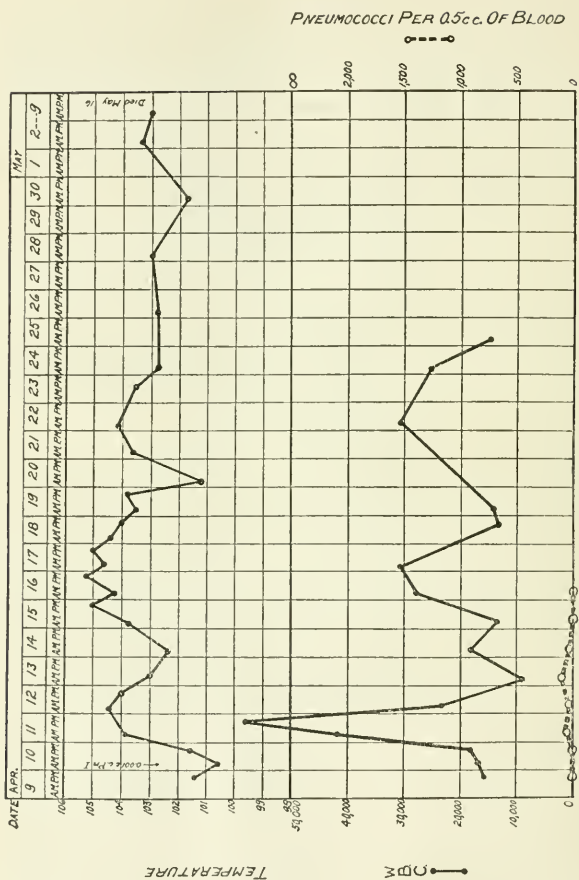
Monkeys 110 and 23 illustrate severe fatal cases of lobar pneumonia with overwhelming pneumococcus septicemia, Monkey 110

dying on the 7th day with complete consolidation of the right lung and a complicating pneumococcus pericarditis, and Monkey 23 dying on the 5th day with a moderately advanced lesion of the left lower lobe. The continued fall of the leucocyte curve with the development of a definite leucopenia coincident with the increasing septicemia has consistently occurred in cases of this nature. During this continued fall in leucocytes examination of stained blood films has shown increasing replacement of mature polymorphonuclear leucocytes by young forms until the former have practically disappeared from the blood. This has been accompanied in some instances by a terminal invasion of the blood by a few nucleated red blood corpuscles and other abnormal cells of the leucocyte series. It is noteworthy that even in the most severe cases of this kind monkeys show little evidence of prostration and maintain the sitting posture until within a relatively short time before death. Monkey 23 shows the unusual feature of a poor febrile reaction, an occurrence that is occasionally seen in rapidly fatal lobar pneumonia in man.

Experiment 7. Monkey 42 (Text-Fig. 7).—Macacus syrichtus, male; weight 4,955 gm. Apr. 9, 1919. Well and active. Apr. 10, 11 a.m. Intratracheal injection of 0.001 cc. (in 1 cc.) of 18 hour broth culture of Pneumococcus Type I. Apr. 11. Appears well. Apr. 12. Moderately sick. Apr. 13. Sick; sits huddled up; breathing rapidly. Apr. 14. Condition the same. Dullness, suppressed breathing, fine moist râles, and pleural friction rub in right lower axilla. Apr. 16. Respirations rapid and labored; coughs occasionally. Dullness, bronchial breathing, and moist râles throughout both axillæ. Apr. 17 to 19. Condition the same. Apr. 20. Appears much better; temperature has fallen, but animal is still breathing rapidly. Apr. 22. Fails to improve. Temperature elevated. Apr. 28. No improvement. Continues to run an irregular fever. Slight exertion causes marked dyspnea. May 9. Animal growing progressively weaker; exhibits constant dyspnea which is greatly increased on exertion. Heart sounds loud and thumping; no murmurs. Dullness and loud bronchial breathing persist over left lower lobe; to and fro crackling râles are heard, probably pleural in origin. No evidence of empyema or pericarditis. May 10 to 15. Condition remains unchanged. May 16. Found lying on floor of cage in state of collapse. Respirations shallow and gasping. 11 a.m. Died.

Autopsy.—Lobar pneumonia, left lower and middle lobes, unresolved; right upper, middle, and lower lobes, nearly resolved; chronic fibrous pleuritis, left and right.

Cultures.—Heart's blood, bronchus, and left lower lobe, *Pneumococcus* Type I.



TEXT-FIG. 7. Monkey 42. Experimental lobar pneumonia, unresolved, following the intratracheal injection of 0.001 cc. of *Pneumococcus* Type I broth culture.

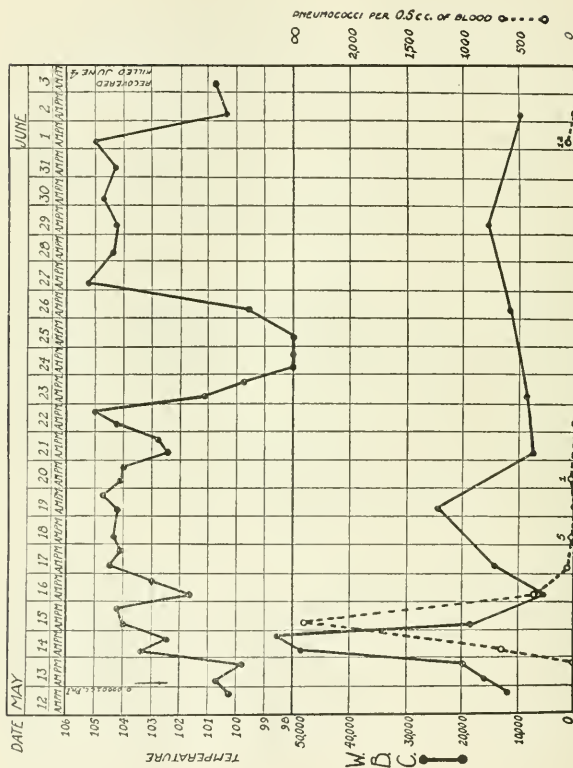
Monkey 42 illustrates a prolonged course due to an unresolved and organizing pneumonia with final death on the 37th day. The characteristic temperature curve with critical fall on the 11th day, and characteristic leucocyte reaction with fall to normal coincident with the peak of the septicemia and secondary rise are well shown. Following apparent recovery from the acute stage of the disease the monkey ran a continuous irregular temperature, manifested extreme weakness and constant shortness of breath on slight exertion, and showed persisting physical signs of consolidation in the left lower lobe. An instructive feature of the autopsy was the finding of pneumococci still present in the unresolved left lower lobe and in the heart's blood, the latter probably being a terminal invasion.

Experiment 8. Monkey 95 (Text-Fig. 8).—Macacus syrichtus, male; weight 4,100 gm. May 12, 1919. Well and active. May 13, 11.05 a.m. Intratracheal injection of 0.00001 cc. (in 1 cc.) of 18 hour broth culture of *Pneumococcus* Type I. May 14. Quiet; respirations moderately accelerated. May 15 and 16. Appears sick; respirations rapid and grunting. May 17. Dullness and suppressed breath sounds over right lower lobe. May 18 to 22. Sick; respirations rapid and labored. May 23. Temperature fallen by crisis; appears much better. May 26. In good condition. May 27. Quiet; temperature elevated. May 28. Appears sick; huddled up in corner of cage; breathing rapidly. May 31. Condition remains the same. No evidence of pericarditis or empyema. June 2. Temperature fallen by crisis; appears better. June 4. Continues well. Killed.

Autopsy.—Lobar pneumonia, left lower lobe; pleuritis, left.

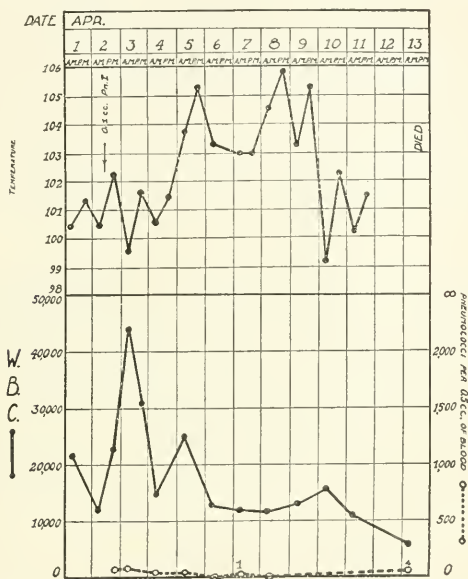
Cultures.—Heart's blood and left lower lobe, no growth.

Monkey 95 presents two unusual features. The first is that of recovery in spite of a very heavy early invasion of the blood stream by pneumococci. This has occurred in few instances. The second is an apparent relapse with a second attack of pneumonia beginning on the 4th day after recovery from the first attack. Examination of the chest during the first attack showed evidence of consolidation in the right lower lobe. It is possible, however, that this was a mistaken diagnosis since autopsy showed only a partially resolved pneumonia of the left lower lobe. There was no evidence of any complication. That the second attack was due to *Pneumococcus* Type I is shown by the isolation of that organism from the blood on June 1.



TEXT-FIG. 8. Monkey 95. Experimental lobar pneumonia, with relapse, following the intratracheal injection of 0.00001 cc. of *Pneumococcus* Type I broth culture.

Experiment 9. Monkey 30 (Text-Fig. 9).—Macacus syrichtus, male; weight 4,120 gm. Apr. 1, 1919. Well and active. Apr. 2, 11.10 a.m. Intratracheal injection of 0.1 cc. (in 1 cc.) of 18 hour broth culture of Pneumococcus Type I. Apr. 3. Appears well. Apr. 4. Moderately sick; breathing rapidly; breath sounds suppressed over left lower lobe. Apr. 5 to 7. Condition the same. Apr. 8. Sicker; respirations rapid and grunting. Examination of chest shows



TEXT-FIG. 9. Monkey 30. Experimental lobar pneumonia following the intratracheal injection of 0.1 cc. of Pneumococcus Type I broth culture. Death from cardiac dilatation (?).

dullness and loud bronchial breathing throughout left axilla, pleural friction rub, moderate dullness, and distant bronchial breathing in right lower front and axilla. Apr. 10. Appears better, but still weak and breathing rapidly. Temperature fallen by crisis. Apr. 11 and 12. Condition the same. Apr. 13, 11.30 p.m. Sudden collapse and death.

Autopsy.—Lobar pneumonia, all lobes; gray hepatization; acute fibrinous pleuritis, bilateral; cardiac hypertrophy and marked dilatation of right auricle and ventricle.

Cultures.—Heart's blood and lungs, no growth.

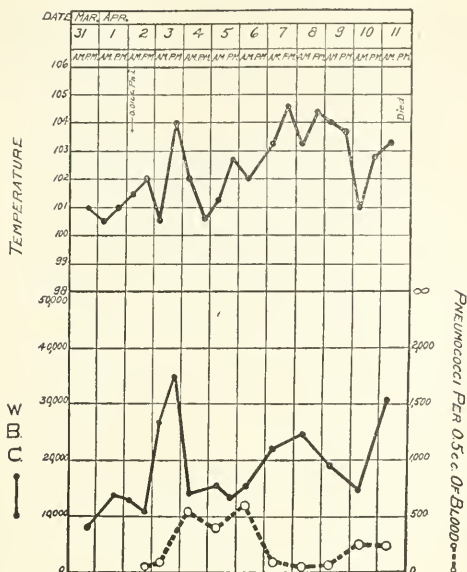
Monkey 30 likewise illustrates two unusual features. The first is an apparent delay in onset of the disease as manifested by symptoms and temperature reaction. It is noteworthy, however, that pneumococci had invaded the blood within 6 hours after intratracheal injection and that the preliminary leucocytosis was completed before there was clinical evidence that the monkey had developed pneumonia. The second is apparent recovery with crisis on the 8th day after inoculation, but death on the 12th day, autopsy revealing extensive consolidation involving all the lobes and marked dilatation of the heart. Cultures from the lungs and heart's blood remained sterile.

Experiment 10. Monkey 31 (Text-Fig. 10).—*Macacus syrichtus*, female; weight 3,850 gm. Apr. 1, 1919. Well and active. Apr. 2, 11.45 a.m. Intratracheal injection of 0.01 cc. (in 1 cc.) of 18 hour broth culture of *Pneumococcus* Type I. Apr. 3. Appears quiet but otherwise well. Apr. 4. Moderately sick; no appetite; respirations rapid. Apr. 6. Sicker; respirations very rapid and grunting. Apr. 8. Condition the same. Dullness, fine moist râles, and bronchial breathing over left lower and middle lobes. Apr. 9. Coughs occasionally. Apr. 10. Condition the same; pleural friction rub in left axilla. X-ray shows a dense shadow in the fourth and fifth left interspaces. Apr. 11. Very sick; respirations deep and gasping. 3 p.m. Died.

Autopsy.—Lobar pneumonia, left upper, middle, and lower lobes; gray hepatization; localized empyema, left; acute purulent mediastinitis.

Cultures.—Heart's blood, bronchus, and empyema, *Pneumococcus* Type I; left lower lobe, no growth.

Monkey 31 represents lobar pneumonia complicated by empyema. The inverse relation between the degree of leucocytosis and the degree of septicemia during the acute stage of the disease is quite striking. A persistent septicemia with rising leucocyte count during the latter part of the disease as shown in this case has nearly always been associated with the development of a complication such as empyema or pericarditis.



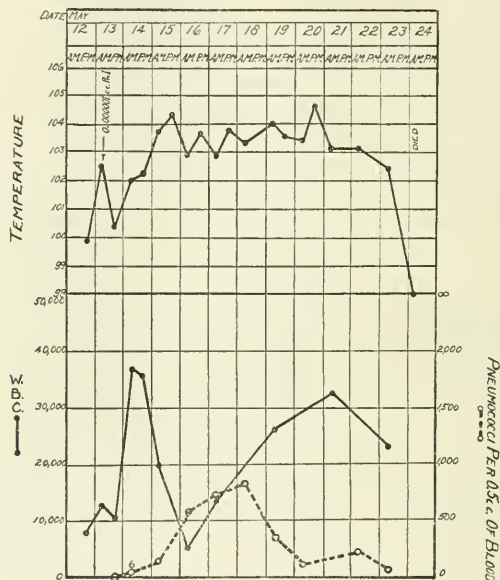
TEXT-FIG. 10. Monkey 31. Experimental lobar pneumonia complicated by empyema following the intratracheal injection of 0.01 cc. of *Pneumococcus* Type I broth culture.

Experiment 11. Monkey 80 (Text-Fig. 11).—Macacus syrichtus, female; weight 2,850 gm. May 12, 1919. Well and active. May 13, 10.35 a.m. Intratracheal injection of 0.000001 cc. (in 1 cc.) of 18 hour broth culture of *Pneumococcus* Type I. May 14. Quiet but otherwise appears well. May 15. Sick; respirations rapid and labored. May 16. Condition the same. May 17. Very sick; breathing rapidly. Abdomen distended and tympanitic. Dullness and diminished breath sounds in left lower axilla. May 18 to 22. Condition the same. May 23. Appears better. May 24. Very sick; lying on floor of cage; respirations rapid and gasping. 11.20 a.m. Died.

Autopsy.—Lobar pneumonia, left lower and middle lobes; gray hepatization; acute fibrinopurulent pericarditis; acute fibrinous pleuritis, left.

Cultures.—Heart's blood, pericardial fluid, left lower lobe, *Pneumococcus* Type I.

Monkey 80 represents a case of lobar pneumonia complicated by a pneumococcus pericarditis. The persisting septicemia with increasing leucocytosis during the latter part of the disease is well shown as in the case of Monkey 31.

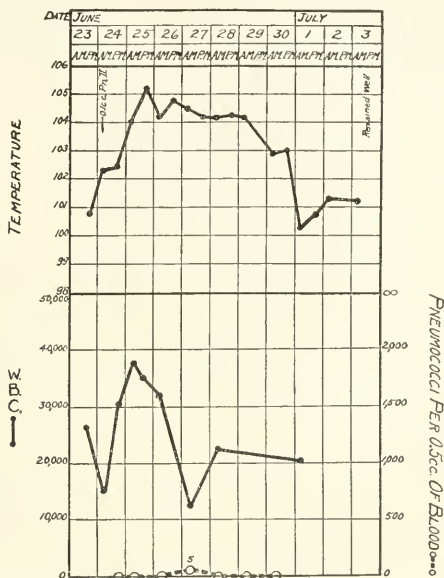


TEXT-FIG. 11. Monkey 80. Experimental lobar pneumonia complicated by fibrinopurulent pericarditis following the intratracheal injection of 0.000001 cc. of *Pneumococcus* Type I broth culture.

Experiment 12. Monkey 48 (Text-Fig. 12).—Macacus syrichtus, male; weight 3,590 gm. June 23, 1919. Well and active. June 24, 9.55 a.m. Intratracheal injection of 0.1 cc. (in 1 cc.) of 18 hour broth culture of *Pneumococcus* Type II. June 25. Quiet; breathing moderately accelerated. June 26. Sick; refuses food; breathing rapidly; coughs occasionally. June 27 to 29. Condition the same. June 30. Appears better; more active. July 1. Temperature fallen by crisis. Appears well and active. July 9. Continues well.

Diagnosis.—Lobar pneumonia.

Monkey 48 presents the typical picture of a mild lobar pneumonia caused by *Pneumococcus* Type II and is analogous in all respects to similar cases produced by the intratracheal injection of *Pneumococcus* Type I.



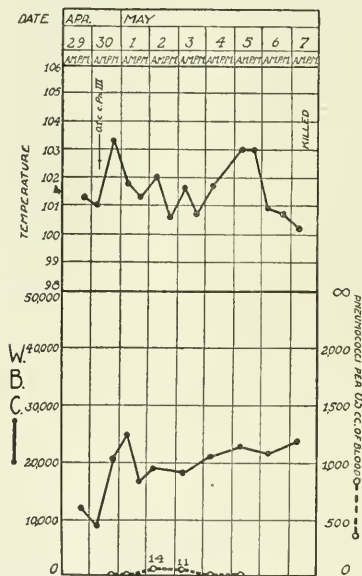
TEXT-FIG. 12. Monkey 48. Experimental lobar pneumonia following the intratracheal injection of 0.1 cc. of *Pneumococcus* Type II broth culture.

Experiment 13. Monkey 82 (Text-Fig. 13).—Cebus capucinus, male; weight 730 gm. Apr. 29, 1919. Well and active. Apr. 30, 10.15 a.m. Intratracheal injection of 0.1 cc. (in 1 cc.) of 18 hour broth culture of *Pneumococcus* Type III. 4.20 p.m. Appears sick; shivering violently; breathing rapidly. May 1. Sick; sitting quietly in corner of cage. Respirations rapid; abdomen distended; fine moist râles heard in right axilla. May 2 and 3. Condition the same. Moderate dullness, moist râles, and distant bronchial breathing over right upper lobe. X-ray shows moderately increased density in the right upper lobe. May 5. Improving. May 7. Appears well and active. Killed.

Autopsy.—Resolving interstitial pneumonia, right upper lobe.

Cultures.—Heart's blood and trachea, no growth.

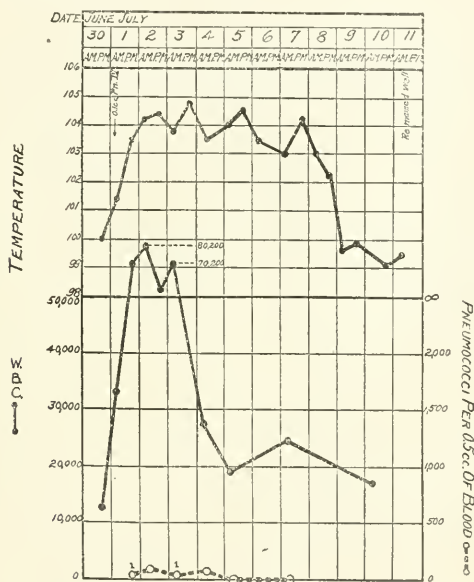
Monkey 82 represents a mild pneumonia due to *Pneumococcus* Type III. The onset was abrupt, with characteristic chill and sharp elevation in temperature and leucocyte count. The temperature curve, however, was irregular and unusual. It has been stated that



TEXT-FIG. 13. Monkey 82. Experimental pneumonia following the intra-tracheal injection of 0.1 cc. of *Pneumococcus* Type III broth culture.

the strain of *Pneumococcus* Type III used was relatively avirulent for monkeys. This is clearly shown in Monkeys 115 and 107 (Table I) both of which ran short courses of 3 and 4 days duration without invasion of the blood by the pneumococcus. It is probable that similar cases which are occasionally seen in man would be diagnosed as

bronchopneumonia or abortive lobar pneumonia. The latter term would seem preferable, since histological examination has shown that invasion of the lung in these monkeys is identical with the mode of invasion in experimental lobar pneumonia, but that further extension of the process to the stage of alveolar exudation and complete lobar consolidation is checked, presumably because of the resistance of the animal against infection with this strain of pneumococcus. This point is discussed further in Paper II.



TEXT-FIG. 14. Monkey 115. Experimental lobar pneumonia following the intratracheal injection of 0.1 cc. of *Pneumococcus* Type IV broth culture.

Experiment 14. Monkey 115 (Text-Fig. 14).—Macacus syrichtus, female; weight 3,015 gm. Previously inoculated with Pneumococcus Type III on June 24, 1919. June 30. Well and active. July 1, 11 a.m. Intratracheal injection of 0.1 cc. (in 1 cc.) of 18 hour broth culture of Pneumococcus Type IV. July 2.

Moderately sick; breathing rapidly. July 3. Sick; no appetite; respirations labored and rapid. July 4 to 8. Condition the same. Coughs occasionally. July 9. Appears well and active. Temperature fallen to normal by crisis. July 16. Continues well.

Diagnosis.—Lobar pneumonia.

Monkey 115 shows that the disease produced by the intratracheal injection of *Pneumococcus* Type IV is identical with that produced by the other types of pneumococci.

Production of Lobar Pneumonia by Contact Infection.

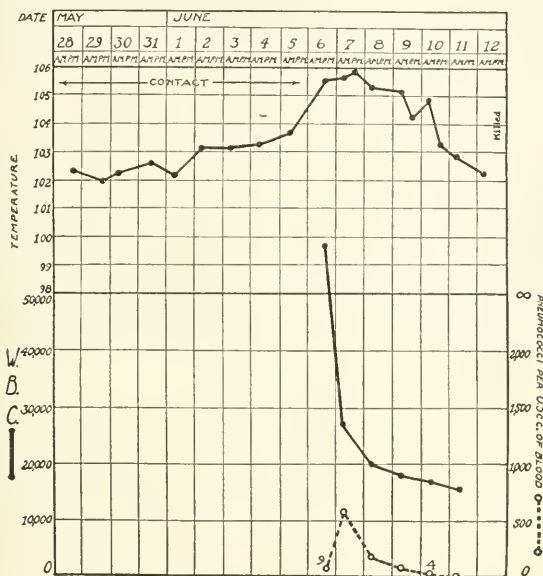
Epidemiological studies by Stillman⁴ have clearly indicated that lobar pneumonia in man caused by *Pneumococcus* Types I and II is probably due in large part to direct or indirect contact infection with other cases of pneumonia caused by these organisms. It was therefore decided to determine whether lobar pneumonia could be experimentally produced in monkeys by contact infection.

Experiment 15.—May 26, 1919. Monkeys 16, 19, 20, 106, 107, and 108 (*Macacus syrichtus*), all well and active, placed in a cage 4 feet by 4 feet by 3 feet. May 27, 10 a.m. Monkeys 109 and 110 (*Macacus syrichtus*) injected intratracheally with 0.001 and 0.000001 cc. of 18 hour broth culture of *Pneumococcus* Type I, respectively, and placed in the same cage. May 28. Monkeys 109 and 110 have developed pneumonia. Contacts appear well. May 30. Contacts remain well. Monkey 109 died. June 1. Monkey 19 has developed spontaneous lobar pneumonia, *Pneumococcus* Type IV; removed from cage.⁵ Other contacts remain well. June 3. Monkey 110 died. Contacts remain well. June 5. Monkey 112 (*Macacus syrichtus*) injected intratracheally with 0.01 cc. of an 18 hour broth culture of *Pneumococcus* Type I, and placed in cage. Contacts remain well. June 6. Monkey 112 has developed pneumonia. 3.45 p.m. Contact Monkey 16 appears sick; breathing rapidly. White blood corpuscles 59,700; blood culture shows *Pneumococcus* Type I (Text-fig. 15). Other contacts well. June 7. Monkey 16 sick; respirations rapid and labored. June 8 to 10. Monkey 16 running typical course of lobar pneumonia. Other contacts remain well. June 10. Monkey 112 died. June 11. Monkey 16 recovered. June 12. Monkey 16 killed. *Autopsy.*—Lobar pneumonia, right lower lobe, resolving. June 20. Contacts remain well. Monkey 114 (*Macacus syrichtus*) injected intratracheally with 0.000001 cc. of an 18 hour broth culture of *Pneumococcus* Type I, and placed in cage. Developed pneumonia and died on June 25. June 26. Contacts remain well.

⁴ Stillman, E. G., *J. Exp. Med.*, 1916, xxiv, 651; 1917, xxvi, 513.

⁵ See Paper III (in press).

The production of lobar pneumonia in Monkey 16 by contact infection eliminated the one minor artificial procedure resorted to in the preceding experiments; namely, the introduction of a needle into the trachea. The fact that only one of the six monkeys exposed contracted *Pneumococcus* Type I pneumonia indicates clearly that



TEXT-FIG. 15. Monkey 16. Spontaneous *Pneumococcus* Type I lobar pneumonia following contact with cases of experimental *Pneumococcus* Type I pneumonia.

other factors besides contact play a part in determining whether or not pneumonia will develop. In contrast with this result the constancy with which lobar pneumonia was produced by the intratracheal injection of even very minute amounts of pneumococcus culture clearly indicates that a virulent pneumococcus once having gained entrance to the respiratory tract below the larynx readily produces pneu-

monia in susceptible individuals. Study of the pathogenesis of experimental lobar pneumonia presented in Paper II indicates that it must penetrate as far as the larger bronchi within the lung before pneumonia will develop. The factors which determine whether or not a pneumococcus will gain access to the lower respiratory tract have not been studied. The following experiment clearly shows that its mere presence in the upper respiratory tract even over considerable periods of time is not sufficient in itself to cause even a highly susceptible animal to develop pneumonia.

Effect of Inoculating Monkeys in the Nose and Throat with Virulent Pneumococcus.

Experiment 16.—June 26, 1919. Monkeys 20, 106, 107, and 108 (*Macacus syrichtus*). All well and active. 10.20 a.m. Nose and throat of each monkey sprayed with 2 to 4 cc. of an 18 hour broth culture of *Pneumococcus* Type I. Monkeys 20, 106, and 108 were held under observation until July 28, Monkey 107 until July 1. All remained perfectly well throughout this period, showing no evidence of pneumonia or of infection of the upper respiratory tract. From time to time saliva was collected and injected intraperitoneally into white mice to determine whether or not *Pneumococcus* Type I was still present. The results are shown in Table II.

TABLE II.
Persistence of Pneumococcus in the Mouth of Normal Monkeys.

Monkey No.	Date of inoculation.	Pneumococcus Type I in mouth.			
		June 30.	July 7.	July 18.	July 28.
	1919				
20	June 26	+	+	+	+
106	" 26	+	+	+	+
107	" 26	+			
108	" 26	+	+	—	+

The continued existence of a virulent *Pneumococcus* Type I in the mouths of monkeys for a period of a month without any of them contracting pneumonia is of interest. In connection with experiments with *Bacillus influenzae* reported in Paper IX it seems advisable to call attention at this point to the fact that a highly virulent pneumococcus inoculated in large amounts into the nose and mouth of animals highly susceptible to pneumococcus infection had absolutely no effect upon them whatsoever.

TABLE III.

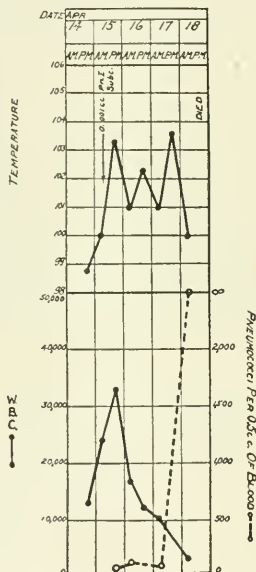
Effect of Intravenous and Subcutaneous Inoculation of Monkeys with Pneumococcus Type I.

Monkey No.	Species.	Weight. gm.	Date of inoculation.	Route of inoculation.	Amount of culture. cc.	Result.	Autopsy.
7	<i>C. capucinus</i> .	1,430	1919 Mar. 7	Subcutaneous.	0.001	Pneumococcus septicaemia. No evidence of pneumonia. X-ray negative. Mar. 14. Recovered.	No autopsy.
46	<i>M. syrichtus</i> .	2,550	Apr. 15	"	0.001	Pneumococcus septicaemia. Apr. 18. Died.	Pneumococcus septicaemia. Lungs normal.
47	"	2,710	" 15	"	0.001	Temporary febrile reaction and leucocytosis of 24 hrs. duration. Blood sterile.	No autopsy.
48	"	3,590	" 15	"	0.001	Temporary leucocytosis. No other apparent effect.	" "
8	<i>C. capucinus</i> .	1,065	Mar. 7	Intravenous.	0.01	Pneumococcus septicaemia. Mar. 8. Died.	Pneumococcus septicaemia. Lungs normal.
49	<i>M. syrichtus</i> .	4,120	Apr. 15	"	0.001	Pneumococcus septicaemia. Apr. 22. Died.	Pneumococcus septicaemia. Lungs normal.
104*	"	1,735	July 8	"	0.001	Temporary pneumococcus septicaemia. July 11. Recovered. July 14. Killed.	No lesions.
105*	"	1,375	" 8	"	0.001	Temporary leucocytosis. No other apparent effect. July 12. Killed.	" "
116	"	1,935	" 8	"	0.001	Pneumococcus septicaemia. No evidence of pneumonia. July 14. Recovered. July 15. Killed.	" "

* Vaccinated with 1 cc. of Pneumococcus Type I saline vaccine (1,000,000,000 pneumococci) on May 22, 29, and June 5, respectively.

Effect of Intravenous and Subcutaneous Inoculation of Monkeys with Virulent Pneumococcus.

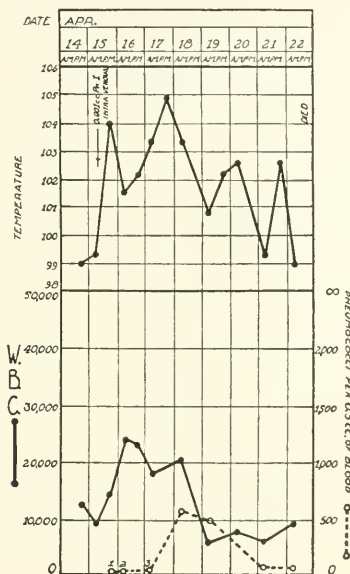
Previous attempts to produce lobar pneumonia in normal animals by intravenous or subcutaneous inoculation of pneumococcus have met with consistent failure.³ In spite of this fact the theory that the



TEXT-FIG. 16. Monkey 46. Effect of the subcutaneous injection of 0.001 cc. of Pneumococcus Type I broth culture.

disease in man is hematogenous rather than bronchiogenic in origin has from time to time been advanced, although without convincing evidence to support it. It therefore seemed advisable to control the results obtained by intratracheal injection by a series of experiments to determine the effect of intravenous and subcutaneous inoculation of pneumococcus in monkeys. Five monkeys received intravenous in-

jections and four monkeys subcutaneous injections of 0.01 to 0.001 cc. of an 18 hour broth culture of *Pneumococcus* Type I, the same strain being used as in the preceding experiments, all with entirely negative results in as far as the production of pneumonia was concerned. The results are shown in Table III. Individual variation in susceptibility probably explains the different results obtained.



TEXT-FIG. 17. Monkey 49. Effect of the intravenous injection of 0.001 cc. of *Pneumococcus* Type I broth culture.

Two illustrative protocols with accompanying clinical charts are presented.

Experiment 17. Monkey 46 (Text-Fig. 16).—*Macacus syrichtus*, female; weight 2,550 gm. Apr. 14, 1919. Well and active. Apr. 15, 10 a.m. Subcutaneous injection of 0.001 cc. (in 1 cc.) of 18 hour broth culture of *Pneumococcus*

Type 1. Apr. 16. Quiet but otherwise appears well. Apr. 17. Appears moderately sick; breathing normally; chest clear. Apr. 18. Sick; respirations slow; chest clear; abdomen slightly distended. Apr. 19. Found dead in morning.

Autopsy.—No lesions; lungs normal; pneumococcus septicemia.

Cultures.—Heart's blood, *Pneumococcus* Type I; lungs, no growth.

Experiment 18. Monkey 49 (Text-Fig. 17).—*Macacus syrichtus*, male; weight 4,120 gm. Apr. 14, 1919. Well and active. Apr. 15, 10.30 a.m. Intravenous injection of 0.001 cc. (in 1 cc.) of 18 hour broth culture of *Pneumococcus* Type I. Apr. 16. Quiet; respirations normal; chest clear. Apr. 18. Moderately sick; no definite symptoms; chest clear. Apr. 19 to 21. Condition the same. Apr. 22. 1 p.m. Very sick; lying on floor of cage. 3 p.m. Died.

Autopsy.—No lesions; lungs normal; pneumococcus septicemia.

Culture.—Heart's blood, *Pneumococcus* Type I.

Monkeys 46 and 49 show that pneumococci injected subcutaneously or intravenously exhibit no tendency to localize in the lungs, or, in fact, anywhere in the body, but produce only a pneumococcus septicemia. Symptoms characteristic of lobar pneumonia were entirely lacking in both animals throughout the course of the infection as they have been in other animals so inoculated.

Effect of Intratracheal Injection of Sterile Broth, Killed Pneumococcus Culture, and Living Avirulent Pneumococcus.

A few further experiments to control the results obtained by intratracheal injection of living virulent pneumococci were carried out as shown in Table IV.

The entirely negative results in these experiments show that the direct intratracheal injection of 1 cc. of fluid with or without pneumococcus cells was not in itself in any way responsible for the development of pneumonia in monkeys injected by this method with living virulent pneumococci.

TABLE IV.
Effect of Intratracheal Injection of Sterile Broth, Killed Pneumococcus Culture, and Living Avirulent Pneumococcus.

Monkey No.	Species.	Weight. gm.	Date of injection.	Material injected.	Result.	Autopsy.
5	<i>C. capucinus</i> .	1,410	1919 Feb. 28	1 cc. of sterile broth.	Slight polymorphonuclear leucocytosis 6 hrs. after injection. No other effect.	No autopsy.
10	"	827	Mar. 18, 10.45 a.m.	1 cc. of Pneumococcus Type I broth culture previously killed by heating at 55°C. for 45 min.	Slight febrile reaction 6 hrs. after injection. Moderate polymorphonuclear leucocytosis 6 hrs. after injection. No other effect. Mar. 19, 3 p.m. Killed.	Lungs appear normal. Microscopic examination shows no abnormalities.
24	"	855	Mar. 20, 10.15 a.m.	2 cc. of 18 hr. broth culture of avirulent strain of Pneumococcus Type I.	No febrile reaction. Blood sterile. Moderate polymorphonuclear leucocytosis 6 hrs. after injection. Mar. 21. X-ray of chest negative. Remained well.	No autopsy.

DISCUSSION.

That the disease experimentally produced in monkeys by the intratracheal injection of minute amounts of pneumococcus cultures is clinically identical with lobar pneumonia in man seems quite clear and requires no special comment. Certain features of the disease, however, which have come out during the course of the study require discussion, since they may serve to throw some light on the course of lobar pneumonia in man.

Although it is generally believed that lobar pneumonia is bronchiogenic in origin there has been no certain evidence that such is the case, as the recurring suggestion that it may be hematogenous in origin testifies. The latter conception has apparently depended upon the clinical observation of cases in which the pneumococcus was shown to be present in the blood at the time of onset of clinical symptoms of pneumonia, or in occasional instances before the clinical symptoms of pneumonia had appeared. That the interpretation of this observation as indicative of the hematogenous origin of lobar pneumonia is incorrect is established by the fact that in the majority of instances in pneumonia experimentally produced by the intratracheal injection of pneumococcus, the organisms have appeared in the blood stream within from 6 to 24 hours after injection, frequently before clinical evidence of pneumonia or elevation of temperature had developed. It is presumable that they rapidly gain access to the blood by way of the lymphatics draining from the lungs, a supposition well supported by histological study of the earliest stages of experimental pneumonia presented in the following paper. The constant production of the disease by intratracheal injection and the constant failure to produce it by other methods of inoculation are believed to be conclusive evidence that infection in lobar pneumonia takes place by way of the respiratory passages.

The failure to produce pneumonia by the instillation of large amounts of a virulent pneumococcus culture into the nose and throat of animals as highly susceptible to pneumococcus infection as monkeys, even though the animals so inoculated continued to carry the pneumococcus in the mouth for at least a month, clearly suggests that there are unknown factors which determine whether or not a

virulent pneumococcus in the upper respiratory tract will gain access to the bronchi. No experiments to determine what these factors are were carried out. It seems established by this observation, however, that the pneumococcus is incapable of initiating an infection of the normal upper respiratory tract in monkeys at least, and that it should probably be regarded only as a secondary invader when found associated with such infections in man.

It is generally stated that the degree of leucocytosis in lobar pneumonia in man is inversely proportional to the severity of the pneumonia, but that individual variation is so great that it is of little prognostic value in the given case. Analysis of the leucocytic reaction during the course of the disease in monkeys, however, has shown a rather surprising constancy in the relation between the leucocyte curve and the severity of the infection as measured by the degree of septicemia, the development of complications, and the final outcome. It has been stated that the onset of pneumonia in monkeys is accompanied by an initial leucocytosis. This preliminary leucocytosis, which was polymorphonuclear in character, usually began within 6 hours after injection and reached its apex within 24 to 48 hours. It varied from 35,000 to 90,000 cells per c.mm., the polymorphonuclear neutrophils comprising from 85 to 95 per cent of the leucocytes. It was not possible to establish any apparent relation between the height of this leucocytosis and the subsequent course of the disease. It was well developed in many cases before clinical evidence of pneumonia or elevation of temperature had occurred, in this respect being similar to the frequent early invasion of the blood by pneumococci. There seems little doubt that it occurred as an immediate response on the part of the animal to invasion of the pulmonary tissue by the pneumococcus and represents an effort on the part of the host to combat the infection at the point of entrance. This was shown to be the case in a monkey killed 3 hours after intratracheal injection (Monkey 72, Paper II), the leucocytes having increased from 15,200 per c.mm. to 25,700 per c.mm. in that time. Histological sections of the right lower lobe showed extensive infiltration of polymorphonuclear leucocytes in the peribronchial tissue near the hilum, many leucocytes passing through the mucosa of the bronchial wall, and many already extruded into the lumen of the bronchus. A

similar though more extensive infiltration of leucocytes was observed in several monkeys dying within 48 hours after inoculation. In some of these considerable phagocytosis of pneumococci had already taken place.

Following the preliminary rise in leucocytes there has been a constant fall in the curve. The rapidity and extent of this fall has seemed to bear a direct relation to the severity of the disease and the degree of pneumococcus invasion of the blood. This is clearly illustrated by contrasting the leucocyte curve of Monkey 115 (Text-fig. 1) with that of Monkey 110 (Text-fig. 5). Study of stained blood films has shown that during this fall mature polymorphonuclear leucocytes are increasingly replaced by young forms with two or three nuclear lobules, until in the very severe cases with overwhelming septicemia and marked leucopenia mature leucocytes have practically disappeared from the blood. In the latter group of cases occasional nucleated red blood corpuscles, myelocytes, and polychromatophilic cells simultaneously appear. This phenomenon points to an initial intense stimulation of the bone marrow followed by a progressive exhaustion as the severity of the infection increases. In harmony with this supposition is the observation that the bone marrow of monkeys dying early with an overwhelming septicemia has frequently been almost entirely devoid of polymorphonuclear leucocytes, and myelocytes have been few in number. It would appear that the leucocytes available at the onset of the disease are transported bodily, so to speak, from the bone marrow to the lungs and that the animal is no longer able to respond with further production of leucocytes in the presence of an overwhelming infection. Whether the progressive exhaustion of bone marrow activity is due to the increasing severity of the infection, or the increasing severity of the infection is due to an inherent weakness of the leucocytic defense in the individual animal is uncertain.

In contrast with the foregoing picture is the one presented by less severe cases which progress favorably to recovery. The leucocyte curve instead of continuing progressively downward turns upward again and progressively rises during the latter half of the disease until crisis occurs, when it again falls to normal. Simultaneously with this increase in leucocytes, the degree of septicemia diminishes until the

blood becomes sterile, usually several days before crisis. This phenomenon has been surprisingly constant and has occurred in nearly all the cases that have recovered. It has been pointed out that a few animals exhibiting this secondary rise in the leucocyte curve have shown a continuing septicemia which terminated in death. In this small group of cases autopsy has shown a complicating pericarditis or empyema in most instances. In both these groups the bone marrow has frequently shown greatly increased numbers of myelocytes.

The mechanism of recovery from lobar pneumonia has been the subject of much investigation. Neufeld and Haendel, Cole and his coworkers, Blake, and others⁶ have attributed it in part to the development of humoral antibodies and have demonstrated the appearance of these antibodies in the circulating blood at or about the time of crisis. More recently Lord⁷ has suggested that another factor may be of considerable importance in bringing about crisis and resolution of the pneumonic process, attributing recovery to local biochemical changes in the course of which the acid death-point of the pneumococcus is reached. Although no study of the mechanism of crisis has been made in the course of the experiments reported, certain isolated observations seem of interest in this connection. In one instance crisis with recovery occurred with pneumococcus septicemia persisting for 48 hours after crisis. In several instances a critical fall in temperature has occurred about the 7th to 9th day with subsequent return of fever and death several days later. Autopsy has shown a resolving pneumonia, death apparently being due to a persisting pneumococcus septicemia usually associated with some complication. In another small group of cases recovery from the general pneumococcus infection has occurred as evidenced by the disappearance of pneumococci from the blood. Crisis, however, did not occur and the monkeys finally died showing an unresolved pneu-

⁶ Neufeld, F., and Haendel, *Arb. k. Gsndhtsamte.*, 1910, xxxiv, 166. Dochez, A. R., *J. Exp. Med.*, 1912, xvi, 665. Avery, O. T., Chickering, H. T., Cole, R., and Dochez, A. R., Acute lobar pneumonia. Prevention and serum treatment, Monograph of The Rockefeller Institute for Medical Research, No. 7, New York, 1917. Blake, F. G., *Arch. Int. Med.*, 1918, xxi, 779.

⁷ Lord, F. T., *J. Exp. Med.*, 1919, xxx, 379.

monia with pneumococci still present in the lung. These observations, though far from proving the fact, are not out of harmony with the theory advanced by Lord with respect to the mechanism of crisis and suggest that other important factors besides the development of humoral immunity are necessary to bring about recovery from pneumonia. With these points in mind it does not seem unreasonable to look upon lobar pneumonia as comprised of two distinct though intimately related processes; one, always present, being the local pulmonary lesion, the other, present in a variable number of cases, being a general infection of the body as manifested by the frequent pneumococcus septicemia. Though ultimate recovery must primarily depend upon the ability of the individual to prevent or terminate the general infection when once established, presumably through the existence or development of a humoral immunity, it does not follow that recovery from the local process with resolution of the pneumonic consolidation need be either coincident with recovery from the general infection or dependent upon the same mechanism. In fact, it would seem well established by numerous clinical observations that recovery from the general pneumococcus infection when it exists usually precedes, by several days at least, recovery from the disease at the time of crisis. On the other hand, certain of the observations cited above would seem to indicate that recovery from the local process as shown by a rapidly resolving pneumonia may occasionally occur prior to recovery from the general infection or even when death from the general infection subsequently takes place. In view of the above considerations it would seem not improbable that at least a dual mechanism may be concerned in bringing about final recovery from lobar pneumonia. Expression of any certain opinion on the subject, however, must depend on much further work.

SUMMARY.

1. Lobar pneumonia has been consistently produced in normal monkeys by the intratracheal injection of minute amounts of pneumococcus culture.
2. The disease produced has been shown to be clinically identical with lobar pneumonia in man.

3. Lobar pneumonia has been produced in the monkey in one instance by experimental contact infection.

4. Normal monkeys inoculated in the nose and throat with large amounts of pneumococcus culture have failed to develop lobar pneumonia though carrying the organism in their mouths for at least a month. They have likewise failed to show any evidence of upper respiratory tract infection.

5. Monkeys inoculated subcutaneously or intravenously with pneumococcus culture have in no instance developed pneumonia, but have either died of pneumococcus septicemia or recovered without localization of the infection in the lungs.

CONCLUSIONS.

1. The pneumococcus is the specific cause of lobar pneumonia.

2. The pneumococcus is unable to initiate an infection of the normal mucous membranes of the upper respiratory tract or to produce pneumonia following intravenous injection, but must gain access to the lower respiratory tract by way of the trachea in order to cause pneumonia.

3. Lobar pneumonia is, therefore, bronchiogenic in origin.

4. Invasion of the blood stream by the pneumococcus in lobar pneumonia is secondary to infection of the lungs.

5. The character of the leucocyte reaction during the course of lobar pneumonia bears a fairly definite relation to the course of the disease.

EXPLANATION OF PLATE 21.

FIG. 1. Monkey 75. Experimental lobar pneumonia; *Pneumococcus* Type I. X-ray of the chest showing consolidation of the left lower lobe.

FIG. 2. Monkey 3. Experimental lobar pneumonia; *Pneumococcus* Type I. X-ray of the chest showing consolidation of the left upper lobe.



FIG. 2.

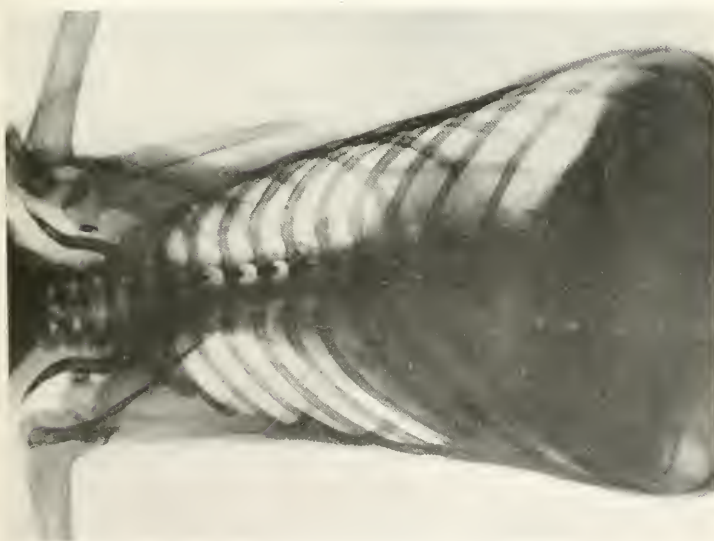


FIG. 1.

STUDIES ON EXPERIMENTAL PNEUMONIA.

II. PATHOLOGY AND PATHOGENESIS OF PNEUMOCOCCUS LOBAR PNEUMONIA IN MONKEYS.

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PLATES 22 TO 44.

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In a preceding paper¹ it has been shown that pneumonia may be produced readily in normal monkeys by the intratracheal injection of minute amounts of pneumococcus culture and that the disease so produced runs a clinical course identical with that of lobar pneumonia in man. In this paper it is proposed, first, to describe the pathology of pneumococcus pneumonia experimentally produced in monkeys, in order to show that the disease is identical with lobar pneumonia in man, and, second, to present observations concerning the pathogenesis of lobar pneumonia based upon study of the pathology of lobar pneumonia in monkeys.

Pathology of Pneumococcus Pneumonia in Monkeys.

Autopsies were performed in 40 cases of pneumonia experimentally produced in monkeys by the intratracheal injection of pneumococcus. Of these, twenty-seven died during the active stage of the disease or later from complications, and thirteen were killed, one on the 9th day of the disease and twelve at varying intervals after recovery by crisis. The cases are summarized in Table I, the fatal cases, including Monkey 26, being arranged according to the duration of the disease, the recovered cases according to the time elapsing between recovery and the killing of the animal for pathological examination. The day of intratracheal injection is considered the 1st day of the disease, since onset of pneumonia had usually occurred within 24 hours after inoculation.

¹ Blake, F. G., and Cecil, R. L., *J. Exp. Med.*, 1920, xxxi, 403.

Macroscopic Pathology.

Macroscopically the lungs at autopsy presented the characteristic picture of lobar pneumonia (Figs. 1 to 3), showing the stages of engorgement, red hepatization, gray hepatization, and resolution. The stage of pneumonia found at autopsy depended primarily upon the duration of the disease at the time of death. This was modified to a certain extent, however, by the amount of pneumococcus culture injected, monkeys receiving a comparatively large amount showing a more advanced involvement early in the disease than those receiving the smaller amounts.

It will be seen from Table 1 that the lungs of monkeys dying within 5 days after intratracheal injection presented the macroscopic picture of engorgement or red hepatization, and that gray hepatization appeared only subsequently to this. In reality, of course, no such sharp distinction could be made between the different stages of the disease, since, as is well recognized,² lobar pneumonia is a progressive process and one stage merges gradually into the next. This was readily observed on examination of the cut surfaces of the lungs and is well illustrated in Fig. 2 in which the right lower lobe shows both red and gray hepatization.

Of the monkeys killed at varying intervals after recovery, five showed an actively resolving pneumonia, two resolution combined with partial organization, and one extensive organization. Organizing pneumonia was also found in two fatal cases (Monkeys 14 and 42) which died at a considerable interval after apparent recovery from the active stage of the disease. The involved lobes in these cases presented a characteristic translucent, grayish appearance, being of a doughy consistency when resolution was occurring, firm and rubbery when organization was taking place.

In three cases of mild pneumonia (Monkeys 82, 96, and 107), in two of which the disease presented the clinical picture of an abortive attack, the lungs showed an interstitial involvement with little or no exudate in the alveoli. In the remaining case (Monkey 75) the pathological picture was complicated by an extensive pulmonary tuberculosis.

² Delafield, F., and Prudden, T. M., A text-book of pathology, New York, 10th edition, 1914, 606.

Tabulation of the lobes involved (Table II) shows that in both the fatal and recovered cases the right and left sides were about equally affected and that the lower lobes were about twice as frequently involved as the middle or upper lobes. The extent of the involvement, however, was in striking contrast in the two groups. Of the twenty-eight fatal cases (including Monkey 26) seventeen showed bilateral pneumonia, while of the twelve recovered cases only three showed involvement on both sides. Eighteen of the fatal cases showed pneumonia of three or more lobes, while none of the recovered cases showed involvement of more than two lobes, and in more than half the pneumonia was confined to one lobe.

The distribution and extent of the pneumonic lesions within the lobe in the different stages of the disease has also been studied. In the earliest stages of engorgement before hepatization had begun the most striking feature was a thickening of the vascular adventitia and walls of the bronchi near the root of the lobe (Figs. 4 to 6). This process spread rapidly throughout the lobe, following the ramifications of the vascular and bronchial trees and appeared always to precede exudation into the alveoli. The interlobular septa were similarly, though less conspicuously, involved. Microscopic examination showed that it was due to edema and leucocytic infiltration of the interstitial tissue. It was accompanied by intense capillary engorgement.

By the time this process had extended throughout the lobe hepatization usually had begun. In monkeys that died early in the stage of red hepatization the consolidation was found invariably to occupy the portions of the lobe proximal to the hilum (Figs. 7 and 8). Surrounding the consolidated areas was a zone of edematous alveoli. In the distal portions of the lobe engorgement was found, the alveoli being still air-containing.

With the further progress of the disease the distal portions of the lobe became progressively consolidated until complete lobar hepatization resulted. By the time red hepatization of the distal portions of the lobe had occurred the portions proximal to the hilum and adjacent to the larger bronchi and vessels were found to have assumed the appearance of gray hepatization (Fig. 2). Finally, in monkeys which died after the 6th day of the disease complete lobar

TABLE I.
Autopsy Findings in Experimental Pneumococcus Lobar Pneumonia in Monkeys.

Monkey No.	Date of inoculation.	Type of pneumococcus.	Amount injected.	Day of disease on which death occurred.	Bacteriology.			Lobes involved and stage of disease.						Complications.
					Heart's blood.	Lungs.	Miscellaneous.	L. U.*	L. M.	L. L.	R. U.	R. M.	R. L.	
2	Feb. 24	I	0.5	1st	Pn. I	R. L., Pn. I.	Br., Pn. I.	R. H.		R. H.	R. H.		R. H.	
5	Mar. 5	I	0.01	1st	"	R. U., "	"			E.	"		"	
1	Feb. 17	I	1.0	2nd	"	R. L., "	"	R. H.		E.	"		"	
3	" 24	I	0.1	2nd	"	L. U., "	"							
29	Apr. 2	I	1.0	2nd	"		Br., Pn. I.	E.		R. H.	"	E.		
109	May 27	I	0.001	3rd	"	R. M., Pn. I.	"	"			"			
6	Mar. 5	I	0.001	4th	"	R. L., "	Tr., "	E.		E.	"	E.		Ac. fib. pericard.
17	" 26	I	1.0	4th	"	"	Pericard., Pn. I.	"		"	"		"	
88	May 20	I	0.000001	4th	"	L. L., "	Br., Pn. I.	"		"	"		"	
98	" 20	I	0.000001	4th	"	"	"	"		"	"		"	
23	Mar. 18	I	1.0	5th	"	"	"	R. H.		"	"		"	
87	May 6	I	0.1	5th	"	"	Br., Pn. I.	R. H.		"	"		E.(C.)	
101	June 20	I	0.000001	5th	"	"	"	E.		E.	"		E.	
114	" 20	I	0.000001	5th	"	R. L., "	"	"		"	"		"	
					S. H.	L. L., S. H.	"			"				
85	May 6	I	0.001	6th	Pn. I	L. L., Pn. I.	S. H.	R. H.		"				Ac. fib. perit.
112	June 5	I	0.01	6th	"	R. L., "	Perit., Pn. I.	"		"		R. H.	R. H.	
					"	"	"					G. H.	G. H.	
110	May 27	I	0.000001	7th	"	"	Br., Pn. I.				R. H.	"	"	Ac. fib.-pur. pericard.
					"	"	Pericard., Pn. I.							Serofib.
83	Apr. 30	I	0.001	8th	"	"	Pl., Pn. I.				E.	"	R. H.	pleurisy.
					"	"	"				R. H.		G. H.	

26	Mar. 21	I 1.0	9th (K.)	N. G.	L. L., N. G. R. L., "	Br., Pn. I. Emp., " Br., "	G. H.	G. H.	G. H.	Emp.; mediast.
31	Apr. 2	I 0.01	10th	Pn. I	L. L., "	Br., "	"	"	"	
100	June 20	I 0.000001	10th	"	"	Br., Pn. I.	"	"	"	E.
67	May 6	I 0.001	11th	"	R. L., Pn. I.	Br., Pn. I.	G. H.	G. H.	G. H.	G. H.
30	Apr. 2	I 0.1	12th	N. G.	" N. G.	"	G. H.	G. H.	G. H.	Hyp. and dil. of heart.
80	May 13	I 0.000001	12th	Pn. I	" Pn. I.	Pericard., Pn. I. Br., Pn. I.	"	G. H.	G. H.	Ac. fib-pur. pericard.
93	" 15	I 0.001	13th	"	R. L., "	"	"	G. H.	G. H.	Hyp. and dil. of heart.
86	" 6	I 0.000001	14th	N. G.	L. L., "	"	"	G. H.	"	"
14	Mar. 26	I 1.0	17th	Pn. I	R. L., N. G.	" N. G.	"	"	"	Org.
42	Apr. 10	I 0.001	37th	"	L. L., Pn. I.	" Pn. I.	Org.	Org.	Res.	Org.
25	" 4	II 0.1	C. 7th K. 7th	N. G.	R. U., N. G.	" N. G.	"	"	"	"
95	May 13	I 0.00001	C. 11th R. 15th C. 21st K. 23rd	"	L. L., "	"	Res. Org.	Res. Org.	"	"

* L. U., L. M., L. L., etc., indicate lobes of the lung. The cardiac lobe is included as part of the right lower lobe. (C.) indicates pneumonia of the cardiac lobe without involvement of the right lower lobe. Pn. I indicates *Pneumococcus* Type I; S. H., *Streptococcus hemolyticus*. Br. indicates bronchus; Tr., trachea; Pl., pleura; N. G., no growth in cultures. E., R. H., G. H., Res., and Org. indicate engorgement, red hepatization, gray hepatization, resolution, and organization, respectively. The stage of pneumonia recorded shows the predominant type of lesion in the lobe as judged by macroscopic examination. K. indicates killed; C., crisis; R., relapse; L., lysis. The other abbreviations are self-explanatory.

TABLE I—*Concluded.*

Monkey No.	Date of inoculation.	Type of pneumococcus.	Amount injected.	Day of disease on which death occurred.	Bacteriology.			Lobes involved and stage of disease.						Complications.
					Heart's blood.	Lungs.	Miscellaneous.	L. U.*	L. M.	L. L.	R. U.	R. M.	R. L.	
82	1919 Apr. 30	III	0.1	C. 7th† K. 8th	N. G		Tr., N. G.				Res.		Res.	
107	July 1	III	1.0	L. 4th† K. 5th	"	R. L., N. G.	Br., "							
96	" 1	III	1.0	L. 3rd† K. 5th	"	L. U., "	" "	Res.			Res.			
64	May 6	I	0.000001	L. 27th K. 30th	"	L. L., Pn. I.	" Pn. I.			Org.				
65	" 6	I	0.1	C. 8th K. 12th	"	R. L., N. G.							Res.	
89	" 20	I	0.001	C. 12th K. 16th	"	" "							"	
7	" 27	IV	0.1	C. 8th K. 13th	"	" "	Br., N. G.			Res.			"	
78	" 15	I	0.001	C. 9th K. 15th	"	" "	" "		Res.				"	
81	" 13	I	0.0001	C. 10th K. 17th	"	" "	" "					Res.	Org. Res.	
75	June 24	II	0.1	C. 8th K. 22nd	"	" "	" "		Res.	Res.				Pulmon. tuberc.

† Interstitial and lobular pneumonia.

‡ Interstitial pneumonia.

consolidation in the stage of gray hepatization was encountered (Figs. 3 and 9).

Pleurisy.—Acute fibrinous pleuritis was almost universally found at autopsy. The fibrinous exudate varied considerably in thickness, depending upon the duration of the disease, and was spread in an easily detachable layer over the surfaces of the involved lobes. In seven cases that died very early there was no evident exudate on the

TABLE II.

Extent and Distribution of Pneumonia in Fatal and Recovered Cases of Experimental Lobar Pneumonia in Monkeys.

	Fatal cases (including Monkey 26).	Recovered cases.
No. of cases.....	28	12
Left lung involved.....	21	6
Right “ “.....	24	9
Bilateral involvement.....	17	3
One lobe involved.....	3	7
Two lobes “.....	7	5
Three or more lobes involved.....	18	0
Left upper lobe involved.....	12	1
Right “ “ “.....	11	3
Left middle “ “.....	12	2
Right “ “ “.....	8	1
Left lower “ “.....	20	4
Right “ “ and cardiac lobes involved.....	23	6
	23	4
	20	3
	43	10

pleura, but microscopic examination showed an early pleurisy. In cases killed after recovery the exudate was undergoing organization.

Complications.—Pneumococcus complications occurred in six cases. Monkey 31 had empyema, Monkey 83 serofibrinous pleurisy, Monkey 17 acute fibrinous pericarditis, Monkeys 80 and 110 acute fibrinopurulent pericarditis, and Monkey 85 localized subdiaphragmatic fibrinous peritonitis. Pneumococci were isolated in pure culture from these complications, which were similar in all respects to like complications of lobar pneumonia in man.

Other Lesions.—Of extrapulmonary lesions the most striking were in the lymph nodes about the hilum and in the bone marrow. The

lymph nodes were consistently enlarged and succulent. Microscopic examination showed that this enlargement was due to distention of the lymph sinuses with numerous large mononuclear cells and lymphocytes, and with moderate numbers of polymorphonuclear leucocytes (Fig. 33). In many instances the large mononuclear cells showed active phagocytosis of polymorphonuclear leucocytes. This process would appear to represent an accumulation of cells brought to the lymph nodes about the hilum by the lymphatic drainage of the lung. Sections of bone marrow taken from the femur showed the widest variation in hematopoietic activity. In some instances the picture was one of almost complete exhaustion, the marrow being entirely devoid of polymorphonuclear leucocytes and largely so of myelocytes (Fig. 34). In others there was marked hyperplasia with greatly increased numbers of leucocytes and myelocytes in the marrow (Fig. 35). All grades of variation between these two extremes were encountered. The picture found depended upon the course of the disease and the degree of infection at the time of death. In monkeys which died during the active stage of the disease with overwhelming pneumococcus septicemia and terminal leucopenia myeloid exhaustion was found. In monkeys which died later without an early overwhelming septicemia but usually with some complication, the picture was one of myeloid hyperplasia. This finding was in keeping with the secondary rise in the leucocyte count exhibited in these cases.

In two animals (Monkeys 30 and 93) marked dilatation and hypertrophy of the heart were found. Both ran a prolonged severe course. Monkey 30 was of particular interest because death occurred suddenly 4 days after apparent recovery by crisis. Autopsy showed nearly complete consolidation of all lobes, cultures from the lungs and heart's blood being sterile. In several other cases running a prolonged severe course, moderate dilatation of the right heart was found, but hardly sufficient to be of certain significance.

Lesions of the abdominal viscera except for cloudy swelling of the liver and kidneys and congestion of the spleen were not encountered.

Microscopic Pathology.

Microscopically, the pneumonia produced in monkeys by the intratracheal injection of pneumococcus likewise showed the characteristic picture of lobar pneumonia in the stages of engorgement, red hepatization, gray hepatization, and resolution or organization. The progressive nature of the process was well shown in monkeys which died at a moderately advanced stage of the disease, in which the microscopic picture of gray hepatization was found in the alveolar tissue proximal to the hilum, of red hepatization in the surrounding zone of tissue, and of engorgement and edema in the more distal portions of the lobe, the peripheral alveoli at the margins most distant from the hilum often being still air-containing.

Engorgement.—As stated above the most striking feature of the stage of engorgement was the perivascular edema and leucocytic infiltration. The walls of the bronchi and the interlobular septa were similarly involved, and extension of the process to the pleura had occurred where the distance from the root of the lobe to the pleura was not great. This interstitial process was particularly conspicuous about the vessels and involved both arteries and veins (Figs. 11, 13, 14, and 15). The peribronchial process (Fig. 12), always less conspicuous than the perivascular one, was usually most extensive in the tissue lying between the bronchus and its accompanying pulmonary artery, this relation being maintained throughout the course of the bronchi to the terminal bronchioles (Fig. 15). The leucocytic infiltration was composed largely of polymorphonuclear leucocytes. Large mononuclear cells and lymphocytes, however, were always present. In a few cases a considerable degree of hemorrhage in the perivascular and peribronchial tissue was found. Fibrin was also present. Necrosis of the tissue was not encountered. The perivascular, peribronchial, and septal lymphatics were distended and often contained large numbers of leucocytes (Figs. 16, 17, and 23).

The epithelium of the bronchi and bronchioles appeared comparatively intact. The bronchi and bronchioles contained little or no exudate (Fig. 15). The vessels and the capillaries of the alveolar walls were greatly engorged (Fig. 18). In the parenchymal tissue

proximal to the hilum the walls of the alveolar ducts and of the adjacent alveoli were often swollen and infiltrated with polymorphonuclear leucocytes, variable numbers of large mononuclear cells, and occasional lymphocytes (Figs. 22 and 23). Some of the alveoli in these areas contained a few large mononuclear cells which appeared to be desquamated alveolar epithelium; otherwise they contained no exudate.

Red Hepatization.—With the further progress of the disease to the stage of red hepatization the more familiar histological pictures of lobar pneumonia appeared. Exudation into the alveoli occurred first in the parenchymal tissue proximal to the hilum, and subsequently progressively involved the more distal portions. In the earliest stages of hepatization the alveoli contained coagulated serum, and the alveolar walls frequently were infiltrated with polymorphonuclear leucocytes and large mononuclear cells. In areas where hepatization was somewhat more advanced the alveoli contained variable numbers of polymorphonuclear leucocytes, large mononuclear cells, and red blood corpuscles embedded in a network of fibrin (Figs. 25, 27, and 28). Frequently the large mononuclear cells contained phagocytosed leucocytes or red blood corpuscles. The character of the mononuclear cells in the exudate and the fact that similar cells were present in considerable numbers in the bronchial walls, perivascular tissue, and alveolar walls during the stage of engorgement, indicate that they were probably, at least in large part, endothelial leucocytes. Some, however, appeared to be desquamated, alveolar epithelial cells.

The number of red blood corpuscles in the exudate varied greatly in different cases and in different areas in the same case. The almost complete absence of red blood corpuscles in the alveolar exudate in some cases in the stage of red hepatization makes it probable that the color of the lung in this stage is largely due to the greatly congested alveolar capillaries.

The interstitial lesions so prominent during the stage of engorgement, though still present in the stage of red hepatization, were much less conspicuous where hepatization had occurred. At the margins of the advancing pneumonic consolidation, however, the primarily interstitial character of the lesion was clearly evident, the peri-

bronchial and perivascular tissue and the alveolar walls being edematous and infiltrated with leucocytes to a variable extent. The lumina of terminal bronchioles and alveolar ducts in many places were still free from exudate, in others where red hepatization was well advanced they contained an exudate similar to that found in the alveoli.

Gray Hepatization.—With still further advance of the process the number of polymorphonuclear leucocytes in the alveolar exudate increased, the fibrin threads often became granular and degenerated, the congestion of the alveolar capillaries disappeared, and the picture passed to that of typical gray hepatization.

The transition from red to gray hepatization occurred first in the portions of the lobe where red hepatization first developed; that is, in the parenchymal tissue proximal to the hilum. Subsequently the more distal portions of the lobe assumed the appearance of gray hepatization. In this stage the alveoli were filled with masses of polymorphonuclear leucocytes. Small numbers of large mononuclears, lymphocytes, and red blood corpuscles were also present. The alveolar walls were thin and appeared well preserved, their capillaries being no longer engorged (Fig. 29). The alveolar ducts and terminal bronchioles were filled with an exudate of leucocytes, red blood corpuscles, and fibrin, and the bronchi contained a variable amount of polymorphonuclear leucocytes and granular debris. The epithelium of the bronchioles and bronchi appeared comparatively intact. Necrosis of the alveolar structure or of the bronchial walls was not found. Edema and leucocytic infiltration of the peribronchial and perivascular tissue, though still present to some extent, were much less conspicuous than in the earlier stages of the disease. The pleura was covered with a dense exudate of fibrin in which polymorphonuclear leucocytes were embedded.

Resolution.—The picture of gray hepatization passed progressively to that of resolution without sharp demarcation between them. In monkeys which died late in the disease or were killed shortly after recovery the alveolar exudate was undergoing rapid disintegration and consisted of leucocytic, nuclear elements and granular debris, occasional lymphocytes, and red blood corpuscles. The alveolar epithelium showed active proliferation and desquamation. As the

process of resolution progressed, desquamated epithelial cells were frequently the predominating cells in the alveoli, the preceding exudate having largely disappeared (Fig. 30). The bronchioles and bronchi were filled with polymorphonuclear leucocytes, mononuclear cells, and desquamated alveolar epithelium in varying proportions. The peribronchial and perivascular tissue was again conspicuous, being infiltrated with large numbers of lymphocytes and plasma cells, and to some extent with polymorphonuclear leucocytes. In still later stages of resolution the alveoli were largely devoid of exudate, containing only here and there a few desquamated epithelial cells. The bronchi and bronchioles no longer showed exudate in their lumina. The perivascular and peribronchial tissue was still conspicuously infiltrated with plasma cells and lymphocytes.

Organization.—Coincident with resolution a variable degree of organization was always found. This was consistently present in the perivascular tissue, though varying greatly in extent, even when complete resolution of the alveolar exudate had taken place. New connective tissue formation about the bronchi also occurred to a considerable extent in cases which showed a widespread organizing pneumonia. Organization of the alveolar exudate, when it occurred, was usually of patchy distribution, alveoli filled with plugs of young connective tissue lying side by side with those in which resolution was rapidly progressing (Fig. 31). The masses of organizing alveolar exudate were covered by a thin layer of epithelium apparently derived from the alveolar walls. In three cases a very extensive organizing pneumonia was found, the normal structure of the lung parenchyma being replaced by young connective tissue in which the distorted remains of alveoli might here and there be found (Fig. 32). The pleural exudate was undergoing organization.

In the following paragraphs autopsy protocols illustrating the different stages of the disease are presented.

PROTOCOLS.

Protocol 1.—Monkey 98. *Macacus syrichtus*, male; weight 4,100 gm. May 20, 1919. Intratracheal injection of 0.000001 cc. of *Pneumococcus* Type I. Developed pneumonia with severe pneumococcus septicemia and died on May 23.

Anatomical Diagnosis.—Lobar pneumonia, left upper, left lower, right upper, and right lower lobes; stage of engorgement.

Pleural cavities.—Contain no fluid. Pleural surfaces are for the most part glistening, but in scattered patches over the upper and lower lobes appear dull. There is no evident deposit of fibrin. *Right lung.*—Somewhat collapsed. The upper lobe feels soggy in its posterior portion, but shows no consolidation. The posterior and inferior surfaces present blotchy dark red areas extending outward from the hilum toward the periphery. The cut surface appears intensely congested and yields a moderate amount of blood-tinged frothy fluid. The walls of the larger vessels and bronchi appear edematous. The alveoli are air-containing. The lower lobe presents a similar picture, the upper, posterior, and external surfaces showing deep red, blotchy areas extending from the hilum toward the periphery. The cut surface shows marked congestion and yields a moderate amount of fluid. The walls of the larger vessels and bronchi appear edematous. The alveoli are air-containing. The bronchi contain a small amount of blood-tinged mucus. The middle lobe is pale pink and crepitant throughout. *Left lung.*—The upper and lower lobes show an involvement similar in character to that found on the right. There is no consolidation. The left middle lobe appears normal. *Bronchial lymph nodes.*—Enlarged and soft; pinkish gray in color. *Other organs.*—Pericardium and heart show no changes. Liver and kidneys show cloudy swelling.

Cultures.—Heart's blood, bronchi, and left lower lobe, *Pneumococcus* Type I.

Macroscopic Examination.—Examination of a large histological section from the right lower lobe (Fig. 4) shows marked thickening of the perivascular tissue. This is particularly conspicuous about the larger arteries and veins near the hilum, but is also present about the smaller vessels in the more peripheral portions of the lobe. The walls of the large and medium sized bronchi are similarly thickened, though much less conspicuously so. The bronchi and bronchioles contain no exudate. Many of the interlobular septa appear moderately thickened. The alveoli are air-containing.

Microscopic Examination.—*Right upper lobe.*—The pleura in sections near the root of the lobe shows capillary engorgement, moderate edema, and infiltration with polymorphonuclear leucocytes, large mononuclear cells, and occasional lymphocytes. This process is especially prominent at the junction of interlobular septa with the pleura. The pleural surface appears intact and there is no fibrinous exudate. The adventitia of the veins and arteries is greatly distended with edema and intensely infiltrated with polymorphonuclear leucocytes and varying numbers of large mononuclear cells and lymphocytes. This process is most conspicuous about the larger vessels, but is also present about the smaller vessels throughout the lobe (Figs. 13 and 14). The perivascular lymphatics are greatly distended and filled with leucocytes (Fig. 17) which in places are embedded in fibrin. The interlobular septa are edematous and infiltrated with abundant polymorphonuclear leucocytes, moderate numbers of large mononuclear cells, and occasional lymphocytes (Fig. 16). Their lymphatics are distended and filled with leucocytes. The walls of the larger bronchi near the root of the lobe

are edematous and infiltrated with leucocytes (Fig. 12). The walls of the smaller bronchi and bronchioles show little or no edema and only moderate leucocytic infiltration (Fig. 15). The epithelium of the bronchi and bronchioles appears intact. The bronchioles contain no exudate. In some of the larger bronchi a few polymorphonuclear leucocytes are seen penetrating between the epithelial cells. The alveoli contain no exudate, except here and there a few large mononuclear cells. The alveolar capillaries are intensely engorged (Fig. 18). Where the engorgement is most extreme the alveoli often appear atelectatic. The alveolar walls show no leucocytic infiltration, except in a few places adjacent to the vessels or bronchi. Moderate numbers of pneumococci are seen in the perivascular and peribronchial tissue, some free, others within polymorphonuclear leucocytes. Occasional pairs are seen in the interlobular septa, in the pleura, and in alveolar walls adjacent to the larger vessels. A few are entangled in the cilia of the epithelium of the larger bronchi. None are seen in the smaller bronchi, bronchioles, or alveolar spaces. *Right lower lobe.*—Presents a similar picture. *Left lung.*—Sections closely resemble those from the right lung. *Bronchial lymph nodes.*—The marginal lymph sinuses are filled with large mononuclear phagocytes, many of which contain polymorphonuclear leucocytes. Many lymphocytes and a few polymorphonuclear leucocytes are also present. No pneumococci are seen.

Protocol 2.—Monkey 23. *Macacus syrichtus*, male; weight 3,555 gm. Mar. 18, 1919. Intratracheal injection of 1 cc. of *Pneumococcus* Type I. Developed pneumonia with pneumococcus septicemia and terminal leucopenia. Mar. 23, 1 a.m. Died.

Anatomical Diagnosis.—Lobar pneumonia, left lower lobe, red hepatization; right lower lobe, engorgement; acute fibrinous pleuritis, left.

Left pleural cavity.—Contains no fluid. There is a thin layer of fresh fibrin over the posterior and external surfaces of the left lower lobe. *Right pleural cavity.*—Contains no fluid. Pleura is smooth and glistening. *Left lung.*—The posterior two-thirds of the left lower lobe from the hilum nearly to the base is firmly consolidated, the surface dark red in color. The anterior portion is crepitant and air-containing. On section the consolidated portion is dark red and finely granular, the alveoli being filled with plugs of exudate. The base and anterior portions are congested, and yield a considerable amount of bloody, frothy fluid. The perivascular tissue appears edematous. The interlobular septa are not prominent. The bronchi contain a moderate amount of bloody mucoid material. The upper and middle lobes are pale pink and crepitant. *Right lung.*—The upper and posterior surfaces of the lower lobe are mottled with deep red patches. The cut surface shows congestion and moderate edema at the root of the lobe. There is no consolidation. The upper and middle lobes appear normal. *Bronchial lymph nodes.*—Enlarged and soft; pinkish gray in color. *Bone marrow of femur.*—Pale pinkish yellow. *Other organs.*—Pericardium and heart show no abnormalities. Liver and kidneys show cloudy swelling.

Cultures.—Heart's blood, left lower lobe, and spleen, *Pneumococcus* Type I.

Microscopic Examination.—*Left lower lobe.*—The pleura is edematous and infiltrated with moderate numbers of polymorphonuclear leucocytes, large mononuclear cells, and occasional lymphocytes. There is a thin meshwork of fibrin on the surface. Sections from the consolidated portion of the lobe show the alveoli uniformly filled with polymorphonuclear leucocytes, moderate numbers of large mononuclear cells, and fibrin. Some contain many red blood corpuscles, others none. The leucocytes are for the most part well preserved; the large mononuclear cells frequently contain phagocytosed leucocytes. The alveolar walls show little or no leucocytic infiltration; the alveolar capillaries are engorged. The adventitia of the arteries and veins is moderately edematous and is infiltrated with many large mononuclear phagocytes and lymphocytes. A few polymorphonuclear leucocytes and an occasional plasma cell are also seen. The perivascular lymphatics are filled with similar cells. The walls of the bronchi and bronchioles are infiltrated with large mononuclear cells, lymphocytes, and moderate numbers of polymorphonuclear leucocytes, their lymphatics being filled with similar cells. The epithelium appears intact; the lumina contain a variable amount of exudate consisting of leucocytes, red blood corpuscles, and fibrin. Sections at the margin of the consolidated area show the alveoli irregularly filled with exudate (Fig. 25). In some, large mononuclear phagocytic cells predominate, in others, the exudate consists almost entirely of red blood corpuscles, while in others, polymorphonuclear leucocytes are abundant. Many contain coagulated serum and fibrin with comparatively little cellular exudate. The alveolar capillaries are intensely engorged. Surrounding this area is a zone in which the alveoli contain only serum and occasional desquamated epithelial cells. The alveolar walls show some infiltration with polymorphonuclear leucocytes and large mononuclear cells. The perivascular tissue is conspicuously infiltrated. The bronchioles contain little or no exudate. Few pneumococci are seen in the consolidated portion of the lobe and, for the most part, they stain poorly and are contained within polymorphonuclear leucocytes of the alveolar exudate. None are seen in the perivascular or peribronchial tissue. At the margins of the consolidated area, however, they are quite numerous in the alveolar exudate and stain well. In the surrounding zone of edematous alveoli they are present both in the serous exudate and in the alveolar walls (Fig. 26); while in the anterior portions of the lobe, where exudation into the alveoli has not occurred, they are seen only in the alveolar walls lying between the capillary and the epithelium lining the alveoli. *Bronchial lymph nodes.*—The marginal lymph sinuses are filled with numerous large mononuclear cells, many of which show inclusions of polymorphonuclear leucocytes and red blood corpuscles. Moderate numbers of lymphocytes, polymorphonuclear leucocytes, and red blood corpuscles are also present (Fig. 33). *Bone marrow of femur.*—Largely devoid of blood cells (Fig. 34). A few myelocytes, lymphocytes, immature polymorphonuclear leucocytes, and megaloblasts are scattered through the tissue or aggregated in small clumps. Groups of normoblasts and an occasional megalocaryocyte are also seen.

Protocol 3.—Monkey 26. *Macacus syrichtus*, male; weight 2,680 gm. Mar. 21, 1919. Intratracheal injection of 1 cc. of *Pneumococcus* Type I. Developed pneumonia. Killed on 9th day while still sick. Leucocytosis at time of death.

Anatomical Diagnosis.—Lobar pneumonia, right and left lower lobes; stage of gray hepatization; acute fibrinous pleuritis, bilateral.

Left pleural cavity.—Contains no fluid; there are fresh fibrinous adhesions between the visceral and parietal pleurae over the left lower lobe. *Right pleural cavity.*—Contains no fluid; there is a fresh fibrinous exudate on the posterior and external surfaces of the right lower lobe. *Left lung.*—There is a massive and uniform consolidation of the left lower lobe involving the entire lobe except the anterior margin which is crepitant. The cut surface is uniformly yellowish gray, finely granular, and comparatively dry. The bronchi and bronchioles are not prominent, but contain a moderate amount of exudate. The upper and middle lobes are collapsed, pale pink, and crepitant throughout. *Right lung.*—The lower lobe shows a massive consolidation similar in all respects to that on the left. The cut surface presents the typical appearance of gray hepatization. *Bronchial lymph nodes.*—Enlarged and succulent. *Bone marrow of femur.*—Of normal consistency; yellowish pink in color. *Other organs.*—Pericardium normal. Heart appears moderately dilated, but otherwise normal. Liver and kidneys show cloudy swelling.

Cultures.—Heart's blood and lungs, no growth; bronchus, *Pneumococcus* Type I.

Microscopic Examination.—*Left lower lobe.*—The pleura is thickened and infiltrated with large numbers of polymorphonuclear leucocytes. The lining pleural cells are well preserved. The pleura is covered with a dense meshwork of fibrin infiltrated with leucocytes. The alveoli, except at the extreme margins of the lobe, are uniformly filled with an exudate of polymorphonuclear leucocytes and fibrin (Fig. 29). A few large mononuclear phagocytic cells and lymphocytes are also seen. The alveolar walls are thin and show little or no leucocytic infiltration. The alveolar capillaries are compressed and empty. The leucocytes of the alveolar exudate are for the most part well preserved, but in portions of the lobe the alveolar exudate is undergoing disintegration, and there is considerable proliferation and desquamation of alveolar epithelium. The adventitia of the vessels, though not distended with edema, is infiltrated with polymorphonuclear leucocytes and moderate numbers of large mononuclear cells and lymphocytes. There is no new connective tissue formation. The walls of the bronchi and bronchioles do not appear thickened, but are everywhere infiltrated with a few polymorphonuclear leucocytes, large mononuclear phagocytes, and lymphocytes. The epithelium is well preserved except for occasional places where desquamation has occurred. The alveolar ducts, terminal bronchioles, and bronchi are filled with an exudate of leucocytes and fibrin. The perivascular and peribronchial lymphatics, though not conspicuously distended, contain many polymorphonuclear leucocytes and moderate numbers of large

mononuclear cells and lymphocytes. The interlobular septa are not conspicuous. In the peripheral alveoli near the pleura the exudate is less compact and frequently shows a preponderance of large mononuclear phagocytic cells with inclusions of leucocytes. The alveolar walls are greatly thickened by infiltration with polymorphonuclear leucocytes. The perivascular tissue is conspicuous and shows a dense leucocytic infiltration, the perivascular lymphatics being filled with leucocytes. Pneumococci are very scanty in the alveolar exudate and when seen stain poorly or are phagocytosed. In the anterior margin where consolidation has not occurred many are seen in the alveolar walls or in the coagulated serum in the alveoli. *Bone marrow of femur*.—Shows a marked cellular hyperplasia. All types of hematopoietic cells are present in abundance, mature forms being very numerous (Fig. 35).

Protocol 4.—Monkey 89. *Macacus syrichtus*, male; weight 2,462 gm. May 20, 1919. Intratracheal injection of 0.001 cc. of *Pneumococcus* Type I. Developed pneumonia and recovered by crisis on May 31. June 4. Killed.

Anatomical Diagnosis.—Lobar pneumonia, right lower lobe; stage of resolution; organizing pleuritis, right.

Left pleural cavity.—Contains no fluid; pleural surfaces glistening. *Right pleural cavity*.—Contains no fluid; the lower portion is obliterated by moderately firm adhesions between the parietal and visceral pleuræ. *Left lung*.—Collapsed, pale pink, and crepitant throughout. *Right lung*.—The lower lobe presents a translucent gray appearance. It is moderately firm throughout but of a doughy consistency. The cut surface presents a fairly uniform pinkish gray gelatinous appearance. The bronchi contain a small amount of mucoid material. The upper and middle lobes are pale pink and crepitant throughout.

Cultures.—Heart's blood and right lower lobe, no growth.

Microscopic Examination.—*Right lower lobe*.—The pleura is covered with a layer of moderately vascular granulation tissue, the interstices of which are infiltrated with numbers of lymphocytes and occasional large mononuclear cells, polymorphonuclear leucocytes, and plasma cells. The pleural lymphatics are filled with similar cells. The alveoli are almost uniformly filled with a pink-staining finely granular material (Fig. 30). The majority contain many large round or oval mononuclear cells with faint staining, sometimes vacuolated protoplasm. A few of these cells contain inclusions of polymorphonuclear leucocytes or red blood corpuscles. Occasional larger multinucleated cells of similar structure are seen. Moderate numbers of lymphocytes are present and an occasional polymorphonuclear leucocyte or red blood corpuscle is also seen. The alveolar epithelium shows active proliferation in many places. The alveolar walls are not infiltrated, their capillaries not engorged. An occasional alveolus contains a plug of organizing exudate. The adventitia of the vessels is conspicuously infiltrated with lymphocytes, moderate numbers of plasma cells, and occasional polymorphonuclear leucocytes. There is a moderate degree of new connective tissue formation about most of the vessels. The walls of the bronchi

and bronchioles are similarly though less conspicuously infiltrated. The lumina contain a small amount of granular material and a few large mononuclear cells similar to those in the alveoli. No pneumococci are seen.

Protocol 5.—Monkey 64. *Macacus syrichtus*, female; weight 2,600 gm. May 6, 1919. Intratracheal injection of 0.000001 cc. of *Pneumococcus* Type I. Developed pneumonia; improving May 13, but continued to run an irregular fever until June 2. June 4. Appeared well. Killed.

Anatomical Diagnosis.—Lobar pneumonia, left lower lobe; organization; fibrous pleuritis, left.

Left pleural cavity.—Contains no fluid; the lower portion is obliterated by moderately firm fibrous adhesions. *Right pleural cavity.*—Contains no fluid; pleural surfaces glistening. *Left lung.*—The lower lobe is firmly consolidated throughout, of translucent gray color, and rubber-like consistency. The cut surface is light gray, dry, and studded with fine, firm granulations. The vessels, bronchi, and interlobular septa are prominent. The upper and middle lobes are pale pink and crepitant. *Right lung.*—Voluminous, pale pink, and crepitant throughout.

Cultures.—Heart's blood, no growth; left lower lobe, four colonies of *Pneumococcus* Type I; left main bronchus, *Pneumococcus* Type I.

Microscopic Examination.—*Left lower lobe.*—The peribronchial and perivascular tissue and the interlobular septa are everywhere greatly thickened by the formation of new connective tissue, the interstices of which are infiltrated with many lymphocytes and plasma cells. In places polymorphonuclear leucocytes are also present. The alveolar walls are similarly affected in many parts of the section, in other places they show no organization. In some areas the process of organization is so extensive that only the distorted and compressed outlines of the alveoli remain. Organization and resolution of the alveolar exudate are going on side by side in adjacent alveoli (Fig. 31). Some alveoli contain numerous desquamated epithelial cells, together with a few lymphocytes and occasional polymorphonuclear leucocytes, in a groundwork of amorphous material. The alveolar epithelium shows very active and widespread proliferation. Other alveoli are filled with plugs of new connective tissue infiltrated with lymphocytes and plasma cells. These plugs are covered with an epithelial layer, the epithelial cells sometimes being cuboidal, sometimes thin and flattened out. A few small groups of alveoli filled with polymorphonuclear leucocytes are also seen. The alveolar ducts and many of the terminal bronchioles are filled with masses of new connective tissue similar to those in the alveoli. The bronchial epithelium appears intact. The bronchi contain polymorphonuclear leucocytes, lymphocytes, and desquamated alveolar epithelial cells in a groundwork of amorphous material.

Pathogenesis of Lobar Pneumonia.

Although the pathology of lobar pneumonia has been well known for many years, little definite knowledge concerning the pathogenesis of the disease has developed. Two principal theories with respect to the initial mode of infection have existed. Some writers³ have advocated the theory that lobar pneumonia is hematogenous in origin, basing this belief on the fact that pneumococci have been isolated from the blood prior to the development of symptoms or physical signs of pneumonia. This point of view, however, has received no experimental support, since attempts to produce lobar pneumonia in animals by intravenous inoculation of pneumococci have consistently failed.^{4, 5, 6} On the other hand, the more commonly accepted view that the mode of infection is by way of the air passages has received a certain amount of confirmation in the experimental production of pneumococcus pneumonia in animals by various methods of intratracheal or intrabronchial inoculation.^{4, 6-9} However, these experiments have not been conclusive, either because the pneumonia produced was not clearly lobar pneumonia or because the method of inoculation or amount of culture material injected tended to vitiate the value of the experiments.

Knowledge concerning the initial point of invasion of the lung by the pneumococcus, the character and location of the primary lesions, and the method by which the infection spreads throughout the lung would appear to be equally doubtful, if one may judge by the indefiniteness with which these subjects are presented in standard textbooks of pathology. Even in the later stages, although it is generally recognized that hepatization in lobar pneumonia is a progressive process, there still remains a difference of opinion as to whether consolidation begins at the periphery and extends centrally or *vice versa*.

³ Kidd, P., *Lancet*, 1912, i, 1589.

⁴ Wadsworth, A., *Am. J. Med. Sc.*, 1904, cxxvii, 851.

⁵ Rasquin, E., *Arch. méd. exp. et anat. path.*, 1910, xxii, 804.

⁶ Armstrong, R. R., *Brit. Med. J.*, 1914, ii, suppl., 57.

⁷ Lamar, R. V., and Meltzer, S. J., *J. Exp. Med.*, 1912, xv, 133.

⁸ Wollstein, M., and Meltzer, S. J., *J. Exp. Med.*, 1912, xvi, 126.

⁹ Winternitz, M. C., and Hirschfelder, A. D., *J. Exp. Med.*, 1913, xvii, 657.

Review of the literature reveals a striking paucity of attempts to answer these questions by experimental procedures. Papers by Müller¹⁰ and by Rasquin⁶ are of particular interest. Müller undertook a study of the pathogenesis of aspiration pneumonia experimentally produced in rabbits by vagotomy. He showed that bacteria-bearing material aspirated from the mouth had penetrated as far as the terminal bronchioles by the end of 6 hours. By 8 hours evidence of bacterial infection was present. The walls of alveoli lying adjacent to the alveolar ducts in which affected bronchioles terminated were swollen and infiltrated with leucocytes, and there were epithelial desquamation and beginning exudation into these alveoli, but the alveolar ducts, atria, and alveoli to which the affected bronchioles led were free from exudate. Bacteria were seen in the infiltrated alveolar walls and in the adjacent alveoli, but not in those with which the affected terminal bronchioles communicated. Still later with wider extension of the process the bacteria were found spreading in the lymph channels of the interstitial framework of the lung and in the alveolar walls, exudation and passage of bacteria into the alveolar spaces occurring only after bacterial invasion of the alveolar walls had occurred. From these observations he inferred that the bacteria gained entrance into the pulmonary tissue at the point where the cuboidal epithelium of the terminal bronchiole gives place to the flattened epithelium of the alveolar duct and atrium, and that the invasion was facilitated by the mechanical injury caused by the aspirated foreign material. He established the fact that further spread of the infection was by way of the interstitial tissue of the lung framework and by way of the alveolar walls.

Rasquin showed that in pneumonia experimentally produced in rabbits by the intratracheal injection of attenuated pneumococci, consolidation invariably occurred in the portions of the lobe proximal to the hilum and usually in the posterior half, the distal portions being free from consolidation. Since the rabbits died with pneumococcus septicemia in a comparatively short time complete lobar consolidation was not found and he considered the process comparable with catarrhal rather than with lobar pneumonia.

In the present study of lobar pneumonia experimentally produced in monkeys by the intratracheal injection of pneumococcus, the attempt has been made to determine the following points concerning the pathogenesis of the disease: (1) whether lobar pneumonia is a bronchiogenic or a hematogenous infection; (2) at what point or points the pneumococcus primarily invades the lung tissue; (3) what the character and location of the initial lesions are; (4) by what paths the pneumococci spread from the initial points of invasion to the remainder of the lobe; and (5) whether hepatization begins more or less simultaneously throughout a lobe or whether it begins centrally and spreads peripherally, or *vice versa*.

¹⁰ Müller, W., *Deutsch. Arch. klin. Med.*, 1902, lxxiv, 80.

Initial Path of Infection.—It has been stated in the preceding paper¹ that pneumonia was consistently produced in normal monkeys by the intratracheal injection of pneumococcus, and it was shown that the pneumonia so produced ran a clinical course identical with that of lobar pneumonia in man. It was furthermore stated that attempts to produce pneumonia by subcutaneous or intravenous inoculation consistently failed. This was equally true of normal monkeys and of monkeys whose resistance to pneumococcus infection had been increased by preliminary inoculations with pneumococcus vaccine. It was also shown that pneumococci may enter the blood stream following intratracheal injection, in some instances before the symptoms or physical signs of pneumonia have developed. It therefore seems safe to conclude that lobar pneumonia is a bronchiogenic and not a hematogenous infection.

Site of Primary Invasion of the Lung Tissue.—It is conceivable that the pneumococcus may primarily invade the lung tissue early in the course of the bronchi by penetration of the bronchial mucous membrane, or that it may pass down the lumina of the bronchi to the terminal bronchioles, and there penetrate the epithelium, or that it may be carried still further and invade the lung tissue only after it has gained access to the alveolar ducts, atria, or alveoli. In an attempt to answer this question a monkey was injected intratracheally with the growth in 10 cc. of an 18 hour broth culture of pneumococcus thrown down by centrifuge and resuspended in 1.5 cc. of the supernatant broth. 3 hours after injection the monkey was killed and autopsy immediately performed. The protocol follows.

Protocol 6.—Monkey 72. *Macacus syrichtus*, female. Apr. 23, 1919, 11 a.m. Intratracheal injection of 10 cc. (in 1.5 cc.) of 18 hour broth culture of *Pneumococcus* Type I. 2 p.m. Killed.

Autopsy.—*Pleural cavities.*—Contain no fluid; pleural surfaces glistening. *Left lung.*—Pale pink and air-containing throughout. No edema or congestion. *Right lung.*—The lower lobe shows an edematous appearing area at the root, the cut surface yielding a moderate amount of fluid. There is no congestion. The remainder of the lobe and the upper and middle lobes appear normal. The larger bronchi contain a moderate amount of frothy fluid. The smaller bronchi are empty. *Lymph nodes.*—Nodes at the hilum appear moderately enlarged. *Other organs.*—Show nothing of importance.

Bacteriology.—Stained films from trachea and right main bronchus show many pneumococci; the bronchial exudate also shows moderate numbers of polymor-

phonuclear leucocytes. There is no phagocytosis. *Cultures*.—Heart's blood, Pneumococcus Type I; trachea, Pneumococcus Type I; right main bronchus, Pneumococcus Type I; left main bronchus, no growth; small bronchi in right and left lower lobes, no growth.

Microscopic Examination.—*Right lower lobe*.—In sections cut close to the hilum the perivascular and peribronchial tissue is edematous and infiltrated with polymorphonuclear leucocytes. The interlobular septa and portions of the pleura are similarly affected. The walls of the smaller bronchi and terminal bronchioles and of their accompanying arteries are densely infiltrated with leucocytes. The walls of the alveolar ducts, atria, and adjacent alveoli are likewise infiltrated (Fig. 22). The epithelium of the large bronchi shows active secretion of mucus but otherwise appears intact in most places. At a number of points, however, the epithelium is injured, and here many polymorphonuclear leucocytes are seen passing into the lumen of the bronchus where they are accumulated in masses on the surface. The bronchioles, alveolar ducts, and atria contain no exudate except in a few instances where the process is well advanced. The alveoli are free from exudate and often appear atelectatic. Moderate numbers of pneumococci are seen entangled in the cilia of the bronchial epithelium or in the mucus secretion. At the points where the bronchial epithelium shows injury pneumococci are seen penetrating the bronchial wall (Fig. 19). Small numbers of pneumococci are seen in the margins of the peribronchial and perivascular tissue, apparently largely in the lymphatics. Pneumococci are more numerous in the walls of the smaller bronchi and vessels, and some are seen in the interlobular septa and affected portions of the pleura. They are present in considerable numbers in the walls of the terminal bronchioles, alveolar ducts, and atria. In the alveolar walls lying adjacent to the vessels, the interlobular septa, and especially the alveolar ducts, they are very abundant, apparently lying between the capillaries and the lining epithelium. None are seen in the lumina of the terminal bronchioles, alveolar ducts, or alveoli, except in a few places where the lesion is well advanced and exudation into the alveoli or alveolar ducts has occurred. In sections from more distal portions of the lobe the tissue appears normal and no pneumococci are seen. *Right upper lobe*.—Sections adjacent to the hilum show a similar picture to that found in the lower lobe. *Other lobes*.—No abnormalities are shown and no pneumococci are seen. *Bronchial lymph node*.—The marginal sinus is crowded with large numbers of polymorphonuclear leucocytes and a few large mononuclear cells and lymphocytes. An occasional free or phagocytosed pneumococcus is seen. *Trachea*.—Appears normal except for a moderately excessive mucus secretion. A few pneumococci are seen entangled in the cilia of the epithelium.

In attempting to form any certain opinion from the results of this experiment as to where the pneumococcus primarily invades the lung tissue, it is obvious that a considerable degree of conservatism is

necessary, since the number of pneumococci injected was very large and since considerable invasion of the tissue had already occurred at the time the animal was killed. It at least may be stated with reasonable positiveness that the initial invasion occurs somewhere comparatively close to the hilum. That it occurred in this case, in part at least, by direct penetration of pneumococci into the walls of the large bronchi is clear. Whether pneumococci had passed down the lumina of the bronchi to the terminal bronchioles or even further into the alveolar ducts and alveoli of the portion of the lobe proximal to the hilum and there invaded the lung tissue is uncertain. It seems doubtful, however, in view of the fact that no pneumococci were found in the lumina of the bronchioles, alveolar ducts, and alveoli, except in a few places where the lesion was well advanced, while on the other hand they were very numerous in the walls of these structures where the process was early and no exudation into the bronchioles or alveoli had occurred. That this practical absence of pneumococci in the terminal bronchioles and alveoli was found in spite of the very large numbers of pneumococci injected lends confirmation to this doubt. The evidence, though strongly against the latter mode of invasion, does not, however, certainly exclude it.

Character and Location of the Initial Lesions.—Opportunity to study the earliest lesions of lobar pneumonia before hepatization had begun was presented in Monkeys 17, 88, 98, 101, 109, and 114, all of which died in the stage of engorgement. The protocol of Monkey 98 has been presented above and is representative of the group. Since all these monkeys, except Monkey 17, were inoculated with minute amounts of pneumococcus culture, such as may be assumed to be reasonably comparable with the probable infecting dose in man, the picture encountered may safely be considered to be that of the earliest stages of lobar pneumonia in man.

Histological sections from all these animals showed clearly that the primary lesions were of the interstitial framework of the lung with accompanying involvement of the lymphatic system. These lesions have been presented in detail above and need no further description. The earliest alveolar lesions beside capillary engorgement were likewise interstitial in character, consisting in leucocytic infiltration of the alveolar walls before exudation into the alveoli

occurred. This took place primarily in the walls of alveoli lying adjacent to terminal bronchioles and alveolar ducts of the central portions of a lobe and to a variable extent in the walls of alveoli lying adjacent to the larger bronchi and vessels.

That the primary lesion of lobar pneumonia is one of the interstitial framework of the lung was well illustrated in Monkeys 96 and 107, which had abortive attacks with recovery on the 3rd and 4th days respectively. Autopsies showed a purely interstitial pneumonia without further advance of the process to the stage of hepatization (Fig. 10).

Spread of the Pneumococci from the Initial Points of Invasion to the Remainder of the Lobe.—This point was readily determined by study of the distribution of pneumococci in the lungs of monkeys dying in the stage of engorgement. As described in the protocols of Monkeys 98 and 72, pneumococci were found in the interstitial tissue and lymphatics of the lung, but were not present in the lumina of the bronchioles, alveolar ducts, or atria, or in the alveolar spaces. In detail, they were present in the perivascular tissue and bronchial walls (Figs. 20 and 21), in the interlobular septa and pleura, in the walls of the terminal bronchioles and alveolar ducts, and in the walls of alveoli lying adjacent to these structures. In more advanced cases of pneumonia in which partial hepatization of a lobe had occurred, a similar interstitial distribution of pneumococci was found in the peripheral unconsolidated portions (Fig. 24). Where beginning exudation had occurred into the alveoli pneumococci were found both in the alveolar walls and in the exudate (Fig. 26). Where hepatization was fully developed they were present in variable numbers in the exudate, often largely phagocytosed or staining poorly, and they were no longer present in the alveolar walls.

It is quite clear, therefore, that invasion and spread of pneumococci in the lung in lobar pneumonia is by way of the perivascular, peribronchial, and septal tissue and lymphatics, and that the alveolar structure is primarily infected by spread of the pneumococci from the grosser framework of the lungs into the alveolar walls. With further advance of the process to the stage of hepatization pneumococci pass out from the alveolar walls into the alveolar spaces together with the exudate.

Development of Hepatization.—It has been stated above and illustrated by the protocol of Monkey 23 that in monkeys which died early in the stage of hepatization the consolidated areas were found invariably to occupy the portions of the lobe proximal to the hilum, while the more distal portions of the lobe were still air-containing (Fig. 8). Although by no means an absolute rule, hepatization usually occurred first in the posterior and upper parts of the lower lobes, in the lower parts of the middle lobes, and in the lower and posterior parts of the upper lobes. This distribution in no way precluded early extension of the consolidation as far as the pleura, since the pleura is comparatively near the large bronchi and vessels in the part of the lobe proximal to the hilum. As the disease progressed hepatization spread to the distal portions of the lobe until complete or nearly complete lobar consolidation had occurred (Fig. 9). As in lobar pneumonia in man, the anterior margins frequently remained air-containing.

Whether a definite lobular arrangement of the exudate might be recognized in the early stages of hepatization has been impossible to determine. It seems possible that a certain amount of variation exists in this respect, depending upon the rapidity with which pneumococci are distributed throughout the lung and the rapidity with which hepatization develops.

Although it seems reasonable to conclude from the study of the development of hepatization in lobar pneumonia in monkeys that consolidation in lobar pneumonia in man begins centrally and spreads peripherally, this does not preclude the probability that in a rapidly spreading pneumococcus invasion of the lung hepatization of an entire lobe may occur within a comparatively short time, nor does it preclude the possibility that in fairly resistant individuals the infection might at first be limited to the interstitial framework in the central portions of the lobe with the primary development of hepatization in the peripheral portions where the framework is less dense. In the latter case, the picture would approach that of a lobular pneumonia or at most a lobar pneumonia of limited involvement.

SUMMARY.

Study of the pathology of pneumonia experimentally produced in monkeys by the intratracheal injection of pneumococcus has shown that it is identical with the pathology of lobar pneumonia in man. It has been found that the pneumococcus primarily invades the pulmonary tissue at some point or points in the portion of the lobe proximal to the hilum, that it spreads rapidly throughout the lobe by way of the perivascular, peribronchial, and septal interstitial tissue and lymphatics, quickly reaching the pleura, and that it invades the alveolar structure primarily by way of the alveolar walls, subsequently passing into the alveolar spaces simultaneously with the outpouring of exudate into the alveoli. It has been shown that the initial mode of invasion may be by direct penetration at one or more points into the walls of the larger bronchi near the hilum. The possibility that primary invasion may occur in terminal bronchioles, alveolar ducts, or alveoli of the parenchyma near the hilum has not been certainly excluded, though the evidence is against this supposition. In harmony with the mode of distribution of pneumococci it has been found that the initial lesions of lobar pneumonia are of the interstitial framework of the lung, with respect both to the grosser framework and to the alveolar framework. Hepatization begins centrally and spreads toward the periphery and is a constantly progressive process. With the development of hepatization the conspicuous interstitial lesions of the earliest stages gradually diminish and are often largely masked when complete lobar consolidation has developed. Resolution is frequently accompanied by a varying degree of organization of the grosser framework of the lung. A variable amount of organization of the alveolar exudate also may occur.

CONCLUSIONS.

1. In lobar pneumonia the pneumococcus primarily invades the lung tissue at some point or points near the root of the lobe.
2. The pneumococcus subsequently spreads throughout the lobe by way of the interstitial framework and lymphatic systems.
3. Lobar pneumonia, therefore, is primarily an interstitial infection of the lung.

4. Consolidation in lobar pneumonia begins in the alveolar tissue proximal to the hilum and progressively spreads to the more distal tissue until complete lobar consolidation develops.

EXPLANATION OF PLATES.

PLATE 22.

FIG. 1. Monkey 83. Lobar pneumonia, *Pneumococcus* Type I; died on 8th day. Posterior aspect of lungs showing lobar consolidation of right middle and lower lobes, partial consolidation of right upper lobe, and normal left lung.

PLATE 23.

FIG. 2. Monkey 83. Lobar pneumonia, *Pneumococcus* Type I; died on 8th day. Cut surface of lungs showing red and gray hepatization of right upper and lower lobes, gray hepatization of right middle lobe, and normal left lung.

PLATE 24.

FIG. 3. Monkey 86. Lobar pneumonia, *Pneumococcus* Type I; died on 14th day. Cut surface of lungs showing gray hepatization of both lower lobes.

FIG. 4. Monkey 98. Lobar pneumonia, *Pneumococcus* Type I; died on 4th day; stage of engorgement. Section from right lower lobe showing thickening of vascular adventitia and of bronchial walls. $\times 3.5$.

PLATE 25.

FIGS. 5 and 6. Monkey 88. Lobar pneumonia, *Pneumococcus* Type I; died on 4th day; stage of engorgement. Sections from left lower lobe and right lower lobe, respectively, showing involvement of interstitial framework of lung. $\times 3.5$.

PLATE 26.

FIG. 7. Monkey 112. Lobar pneumonia, *Pneumococcus* Type I; died on 6th day; stage of engorgement and red and gray hepatization. Section from left lower lobe showing beginning peribronchial consolidation. $\times 3.5$.

FIG. 8. Monkey 29. Lobar pneumonia, *Pneumococcus* Type I; died on 2nd day; stage of engorgement and red hepatization. Section through left lower lobe showing consolidation of central portion with engorgement and edema of peripheral portions.

PLATE 27.

FIG. 9. Monkey 93. Lobar pneumonia, *Pneumococcus* Type I; died on 13th day; stage of gray hepatization. Section through right lower lobe showing uniform consolidation except at anterior margin. $\times 3.5$.

FIG. 10. Monkey 107. Abortive lobar pneumonia, *Pneumococcus* Type III; recovered by lysis on 4th day; killed on 5th day. Section through right lower lobe showing limitation of involvement to interstitial framework of lung. $\times 3.5$.

PLATE 28.

FIG. 11. Monkey 17. Lobar pneumonia, *Pneumococcus* Type I; died on 4th day; stage of engorgement. Edema and leucocytic infiltration of adventitia of large pulmonary vein. $\times 100$.

PLATE 29.

FIG. 12. Monkey 98. Lobar pneumonia, *Pneumococcus* Type I; died on 4th day; stage of engorgement. Edema and leucocytic infiltration of wall of large bronchus. $\times 100$.

FIG. 13. Monkey 98. Lobar pneumonia, *Pneumococcus* Type I; died on 4th day; stage of engorgement. Perivascular edema and leucocytic infiltration of a small peripheral vein. $\times 100$.

PLATE 30.

FIG. 14. Monkey 98. Lobar pneumonia, *Pneumococcus* Type I; died on 4th day; stage of engorgement. Leucocytic infiltration about a small vein. $\times 400$.

PLATE 31.

FIG. 15. Monkey 98. Lobar pneumonia, *Pneumococcus* Type I; died on 4th day; stage of engorgement. Bronchiole and pulmonary arteries showing leucocytic infiltration of walls with absence of exudate in bronchiole and adjacent alveoli. $\times 100$.

FIG. 16. Monkey 98. Lobar pneumonia, *Pneumococcus* Type I; died on 4th day; stage of engorgement. Edema and leucocytic infiltration of interlobular septum. Lymphatics filled with polymorphonuclear leucocytes. $\times 100$.

PLATE 32.

FIG. 17. Monkey 98. Lobar pneumonia, *Pneumococcus* Type I; died on 4th day; stage of engorgement. Distended lymphatic filled with polymorphonuclear leucocytes.

FIG. 18. Monkey 98. Lobar pneumonia, *Pneumococcus* Type I; died on 4th day; stage of engorgement. Capillary engorgement and atelectasis of alveoli. $\times 400$.

PLATE 33.

FIG. 19. Monkey 72. Killed 3 hours after intratracheal injection of *Pneumococcus* Type I. Invasion of wall of large bronchus by pneumococci.

PLATE 34.

FIG. 20. Monkey 88. Lobar pneumonia, *Pneumococcus* Type I; died on 4th day; stage of engorgement. Pneumococci in wall of large bronchus, right lower lobe. $\times 1,000$.

FIG. 21. Monkey 88. Lobar pneumonia, *Pneumococcus* Type I; died on 4th day; stage of engorgement. Pneumococci in perivascular tissue of small pulmonary vein near periphery. $\times 1,000$.

PLATE 35.

FIG. 22. Monkey 72. Killed 3 hours after intratracheal injection of *Pneumococcus* Type I. Early alveolar lesion showing polymorphonuclear leucocytic infiltration of walls of alveolar duct and adjacent alveoli with absence of exudate in duct and alveoli. $\times 100$.

PLATE 36.

FIG. 23. Monkey 5. Lobar pneumonia, *Pneumococcus* Type I; died on 1st day; stage of engorgement and red hepatization. Leucocytic infiltration of alveolar walls beyond the margin of the consolidated area; also distention of perivascular lymphatics. $\times 100$.

PLATE 37.

FIG. 24. Monkey 6. Lobar pneumonia, *Pneumococcus* Type I; died on 4th day; stage of engorgement and red hepatization. Section from periphery of right lower lobe showing pneumococci in alveolar walls.

PLATE 38.

FIG. 25. Monkey 23. Lobar pneumonia, *Pneumococcus* Type I; died on 5th day; stage of red hepatization. Margin of consolidated area showing alveoli containing varying proportions of serum, red blood corpuscles, fibrin, polymorphonuclear leucocytes, and large mononuclear cells. $\times 100$.

PLATE 39.

FIG. 26. Monkey 23. Lobar pneumonia, *Pneumococcus* Type I; died on 5th day; stage of red hepatization. Section from margin of consolidated area showing pneumococci in alveolar walls and in serous exudate in alveoli.

PLATE 40.

FIG. 27. Monkey 110. Lobar pneumonia, *Pneumococcus* Type I; died on 7th day; stage of red and gray hepatization. Alveolar exudate of coagulated serum, fibrin, polymorphonuclear leucocytes, and large mononuclear cells. $\times 400$.

PLATE 41.

FIG. 28. Monkey 112. Lobar pneumonia, *Pneumococcus* Type I; died on 6th day; stage of red and gray hepatization. Alveolar consolidation. $\times 100$.

FIG. 29. Monkey 26. Lobar pneumonia, *Pneumococcus* Type I; killed on 9th day; stage of gray hepatization. Alveoli filled with exudate of polymorphonuclear leucocytes. $\times 100$.

PLATE 42.

FIG. 30. Monkey 89. Lobar pneumonia, *Pneumococcus* Type I; killed 4 days after recovery by crisis. Resolution of alveolar exudate. $\times 100$.

FIG. 31. Monkey 64. Lobar pneumonia, *Pneumococcus* Type I; killed on 30th day. Organization and resolution. $\times 100$.

PLATE 43.

FIG. 32. Monkey 14. Lobar pneumonia, *Pneumococcus* Type I; died on 17th day. Organization. $\times 100$.

FIG. 33. Monkey 23. Lobar pneumonia, *Pneumococcus* Type I; died on 5th day. Marginal sinus of bronchial lymph node filled with large mononuclear phagocytes, polymorphonuclear leucocytes, and lymphocytes. $\times 400$.

PLATE 44.

FIG. 34. Monkey 23. Lobar pneumonia, *Pneumococcus* Type I; died on 5th day. Bone marrow of femur showing almost complete exhaustion of hemopoietic cells. $\times 400$.

FIG. 35. Monkey 26. Lobar pneumonia, *Pneumococcus* Type I; killed on 9th day. Bone marrow of femur showing hyperplasia. $\times 400$.

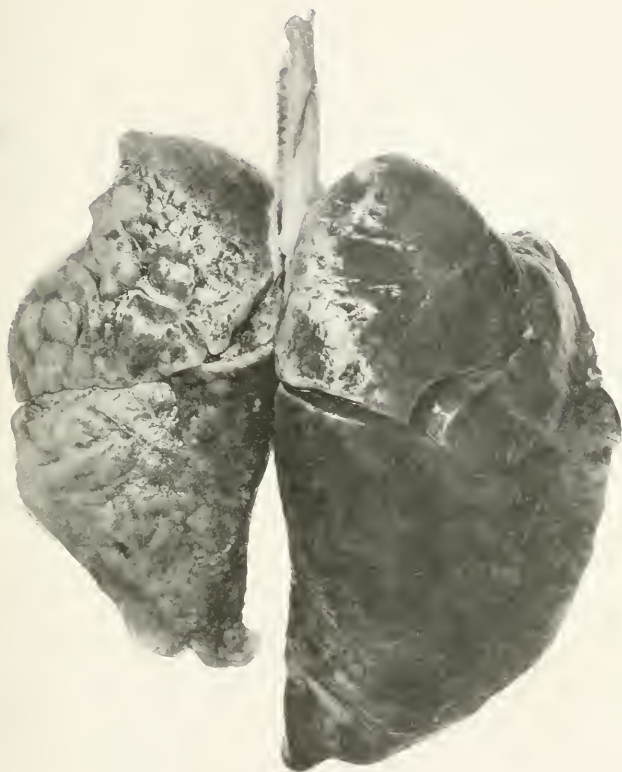


FIG. 1.

(Blake and Cecil: Experimental pneumonia. II.)



FIG. 2.

(Blake and Cecil: Experimental pneumonia. II.)



FIG. 3.

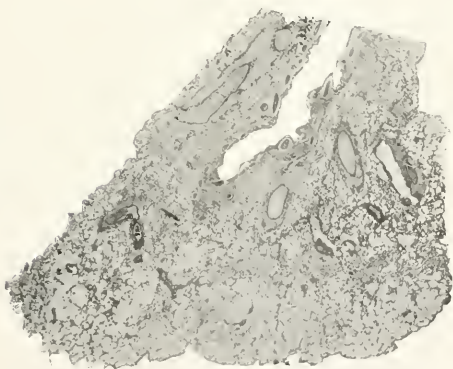


FIG. 4.

(Blake and Cecil: Experimental pneumonia. II.)

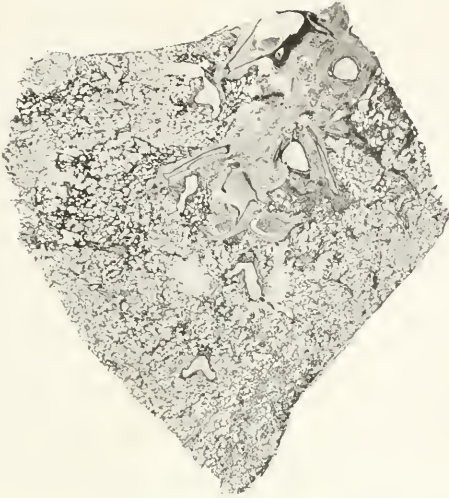


FIG. 5.

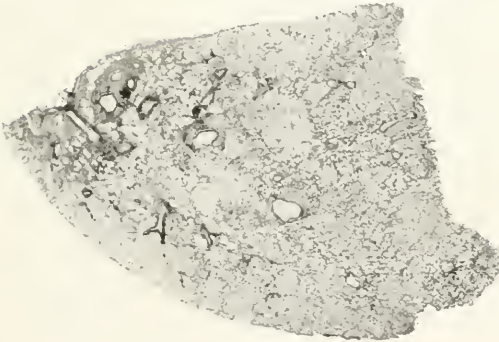


FIG. 6.

(Blake and Cecil: Experimental pneumonia. II.)



FIG. 7.

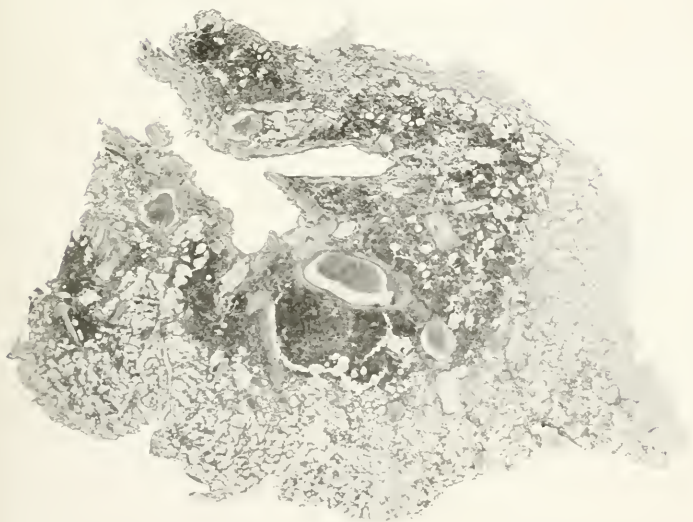


FIG. 8.

(Blake and Cecil: Experimental pneumonia. II.)

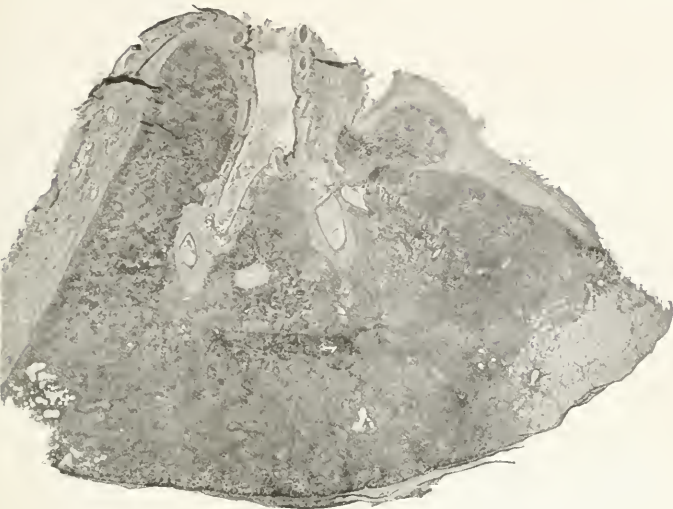


FIG. 9.



FIG. 10.

(Blake and Cecil: Experimental pneumonia. II.)

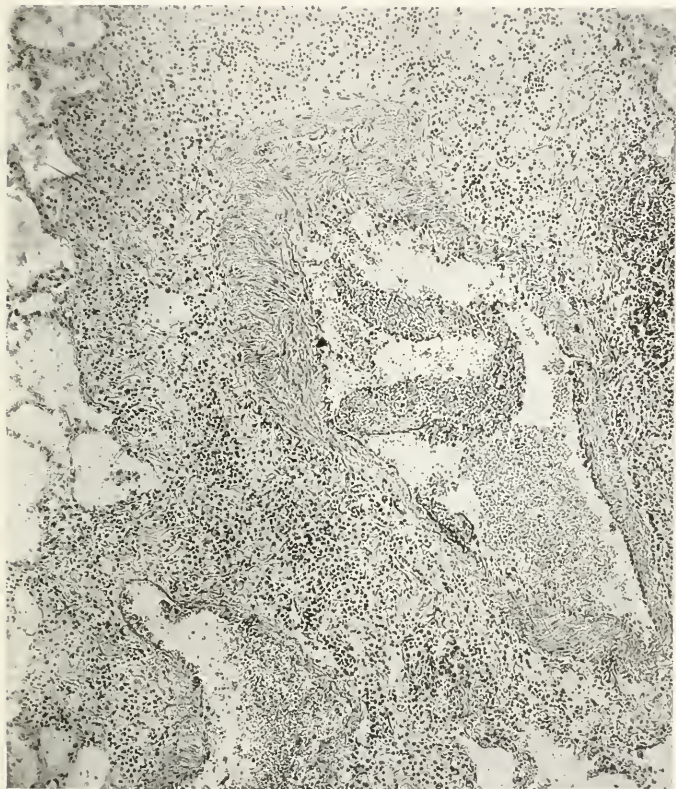


FIG. 11.

(Blake and Cecil: Experimental pneumonia. II.)

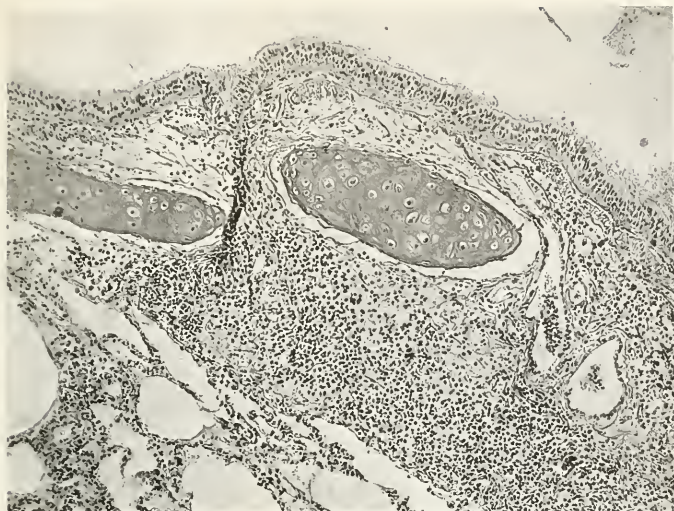


FIG. 12.



FIG. 13

(Blake and Cecil: Experimental pneumonia. II.)

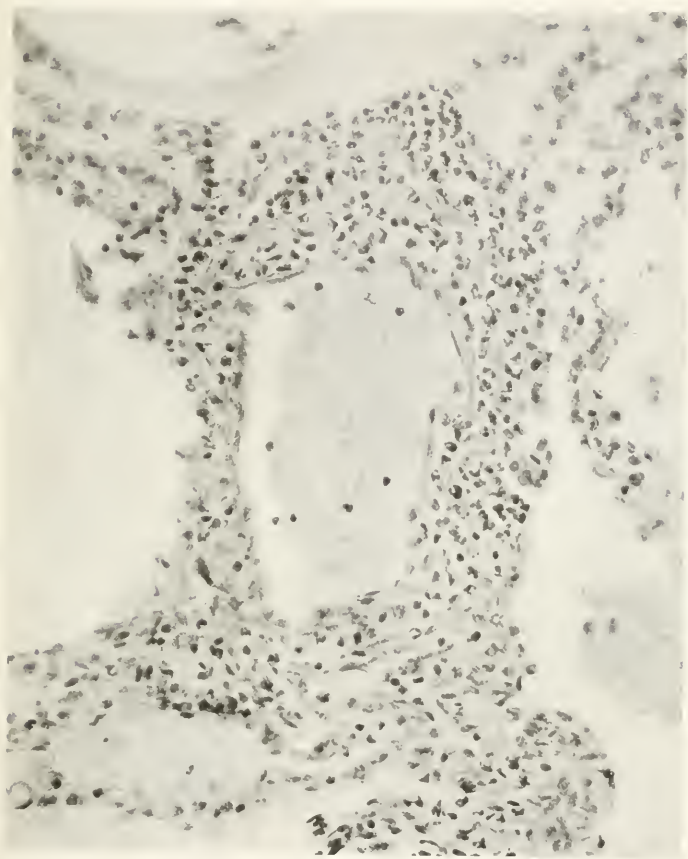


FIG. 14.

(Blake and Cecil: Experimental pneumonia. II.)

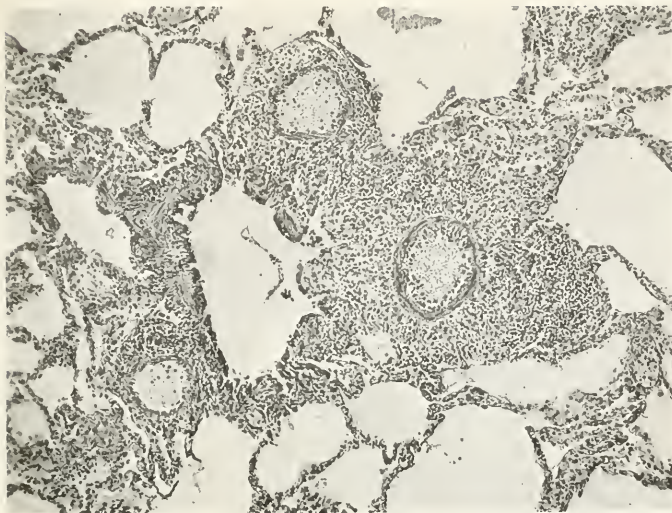


FIG. 15.

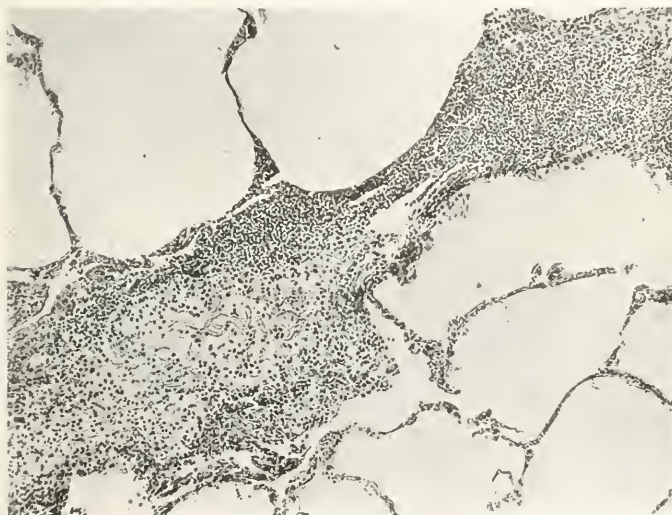


FIG. 16.

(Blake and Cecil: Experimental pneumonia. II.)

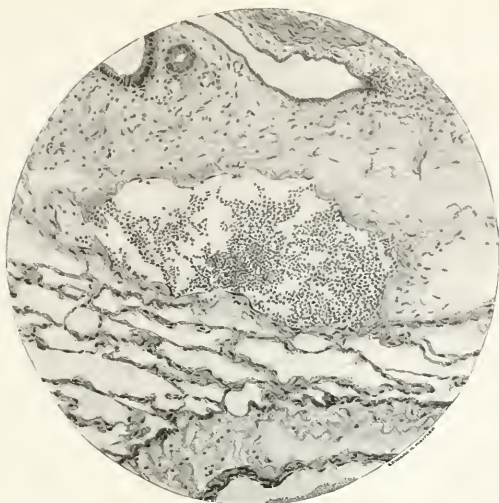


FIG. 17.

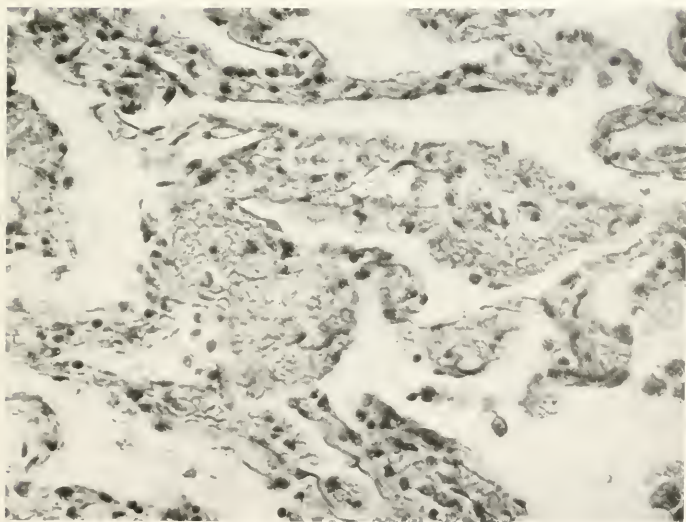


FIG. 18.

(Blake and Cecil: Experimental pneumonia. II.)

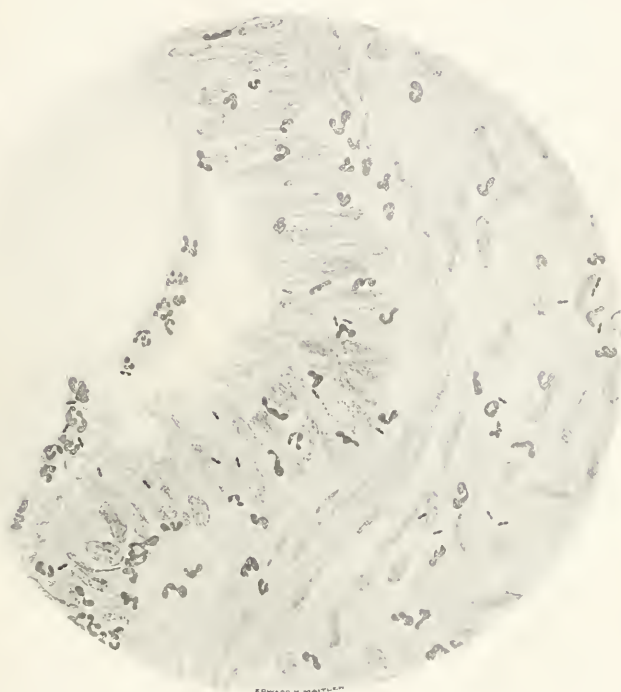


FIG 19.

(Blake and Cecil: Experimental pneumonia. II.)

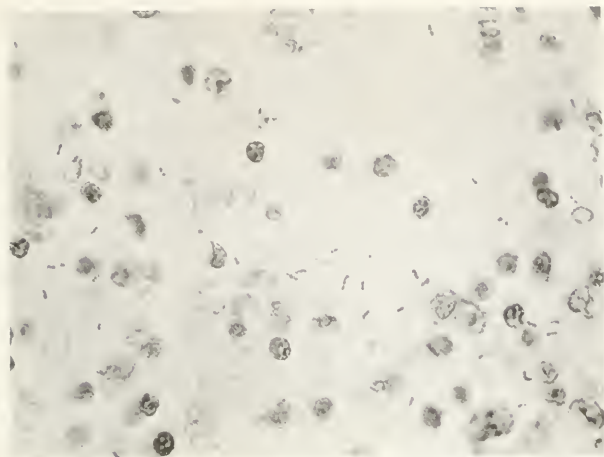


FIG. 20.

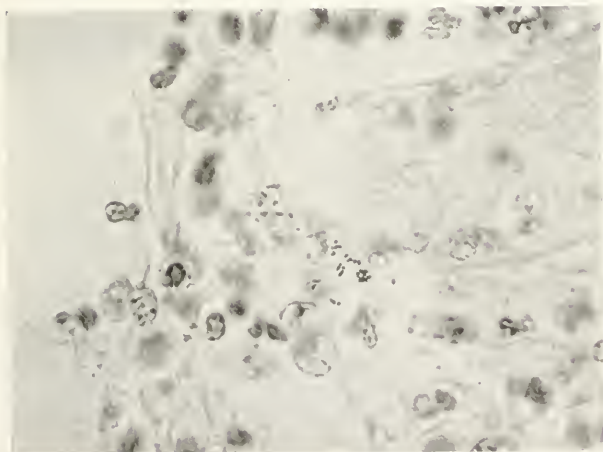


FIG. 21.

(Blake and Cecil: Experimental pneumonia. II.)

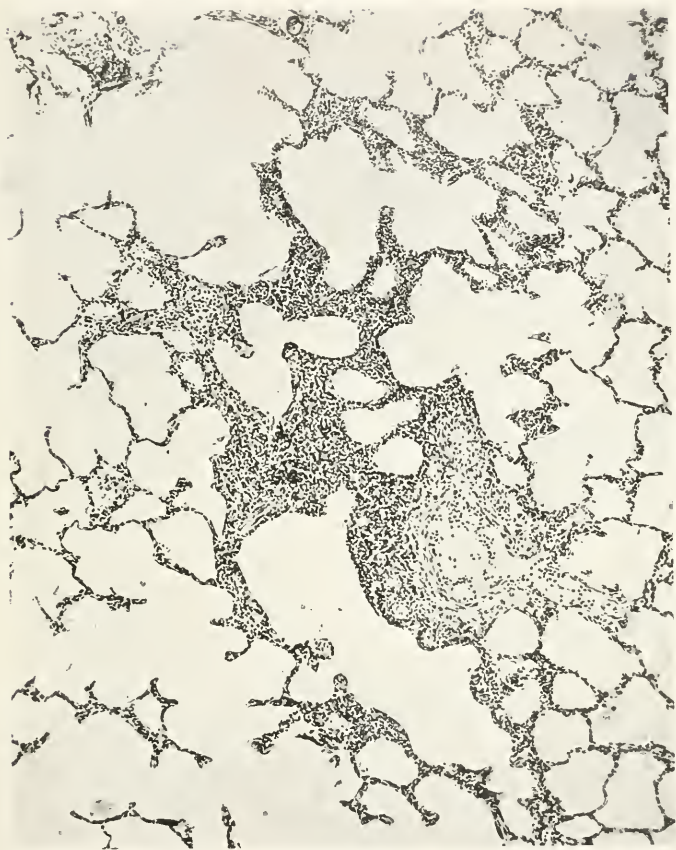


FIG. 22.

(Blake and Cecil: Experimental pneumonia. II.)

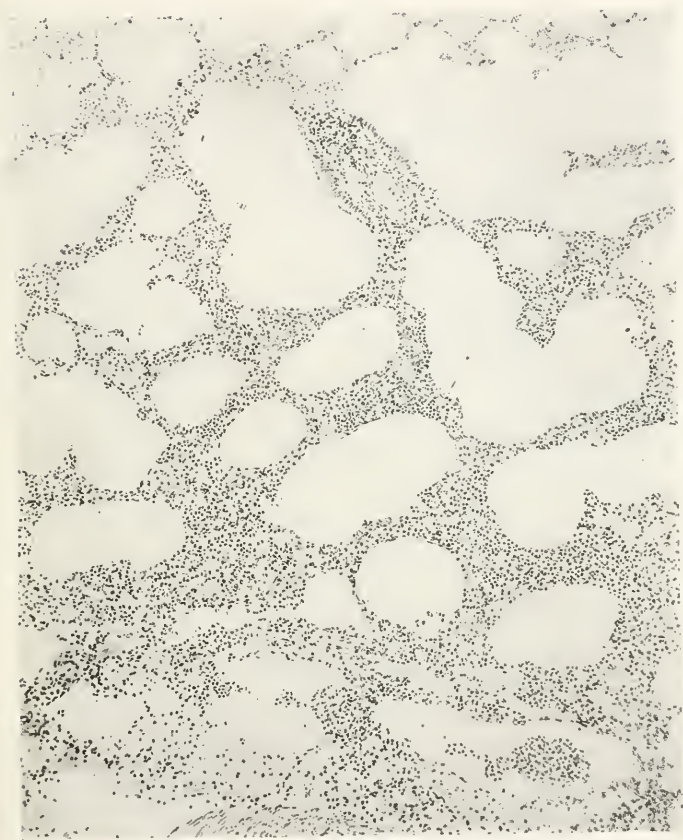


FIG. 23.

(Blake and Cecil: Experimental pneumonia. II.)

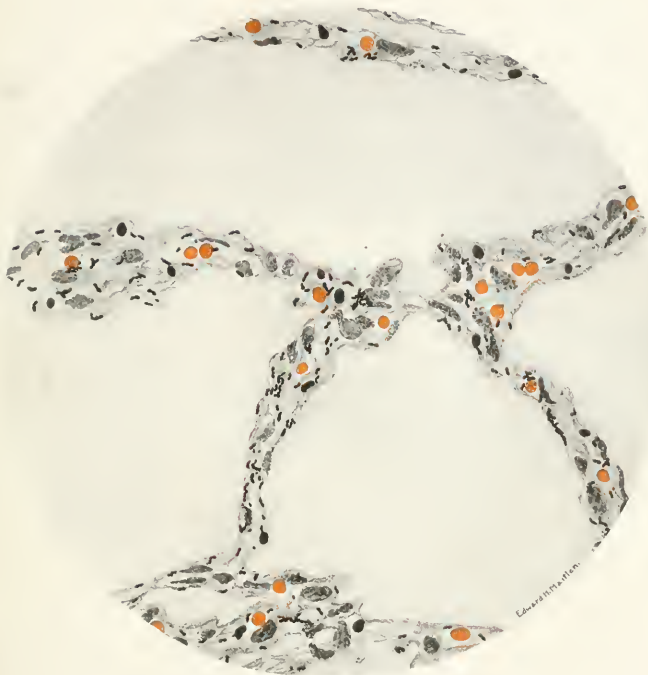


FIG. 24.

(Blake and Cecil: Experimental pneumonia. II.)

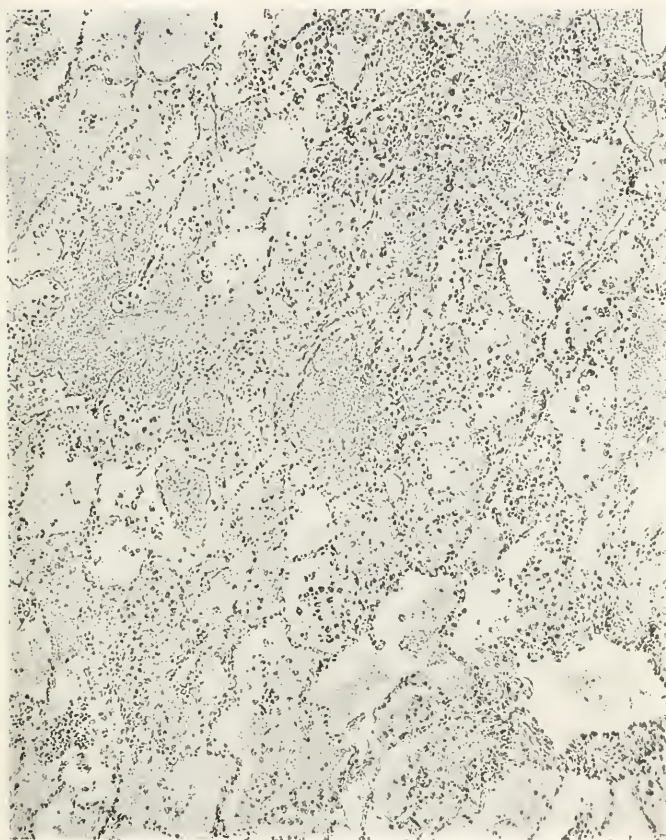


FIG. 25.

(Blake and Cecil: Experimental pneumonia. II.)

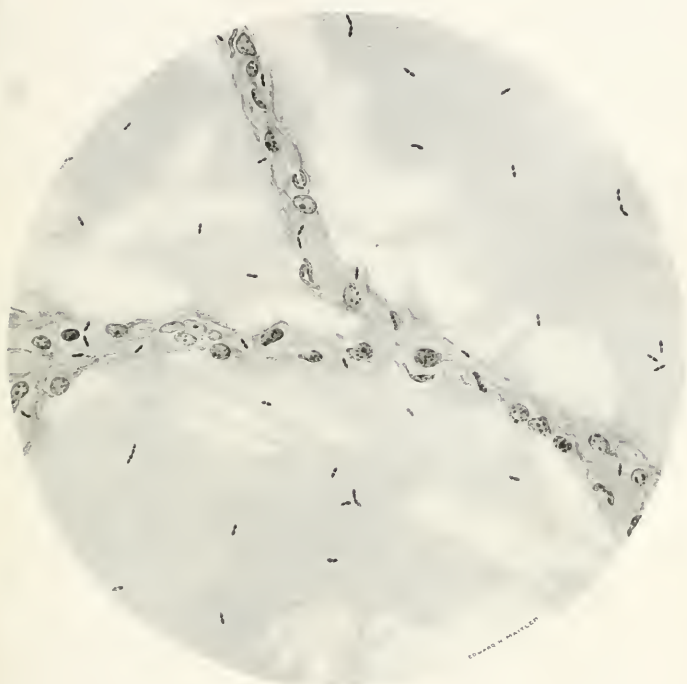


FIG. 26

(Blake and Cecil: Experimental pneumonia. II.)

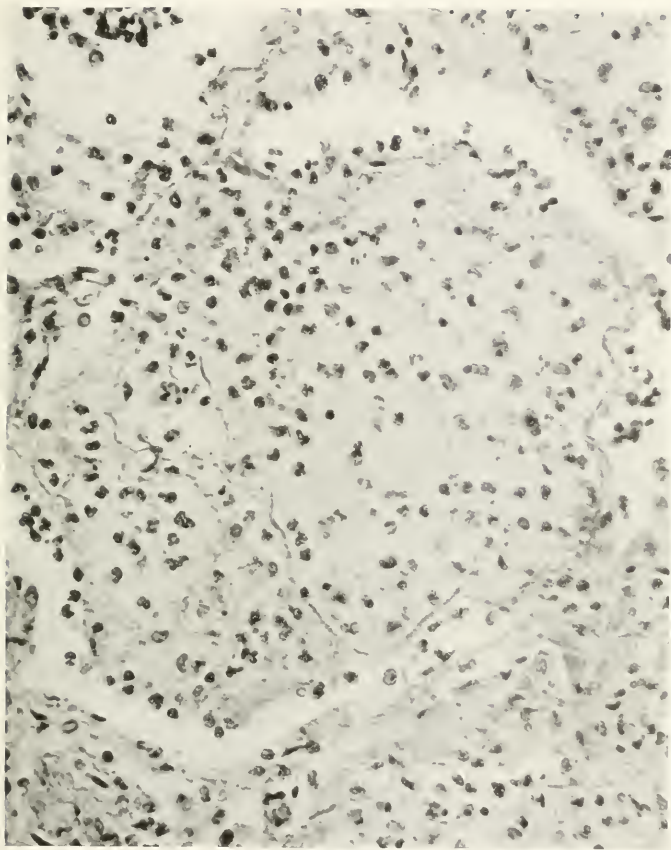


FIG. 27.

(Blake and Cecil: Experimental pneumonia. 11.)

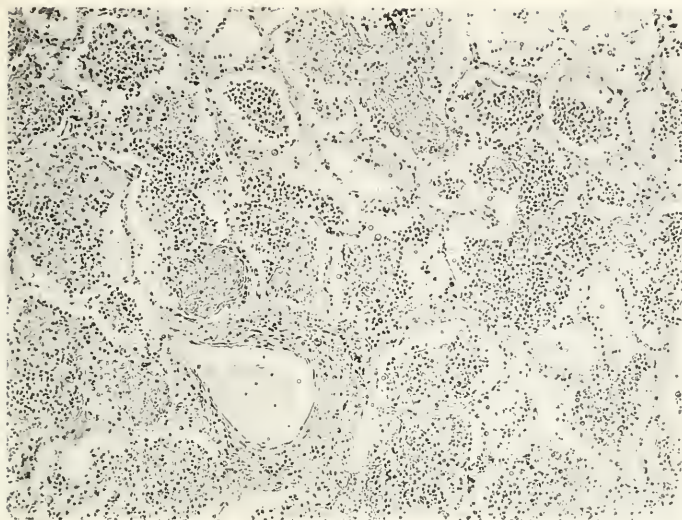


FIG. 28.

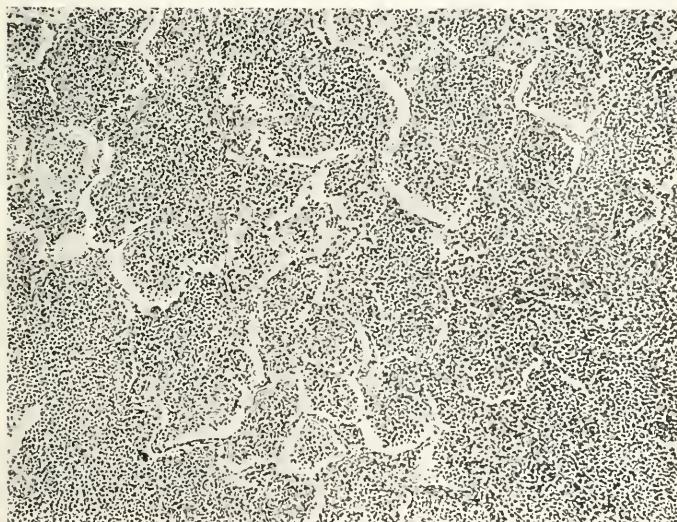


FIG. 29.

(Blake and Cecil; Experimental pneumonia. II.)

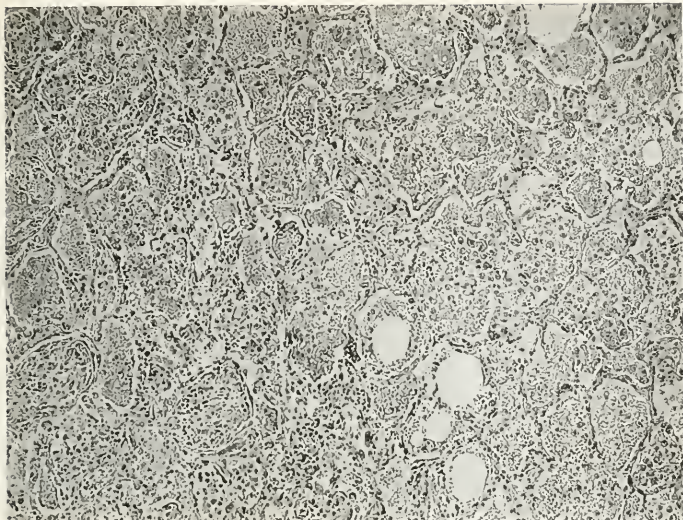


FIG. 30

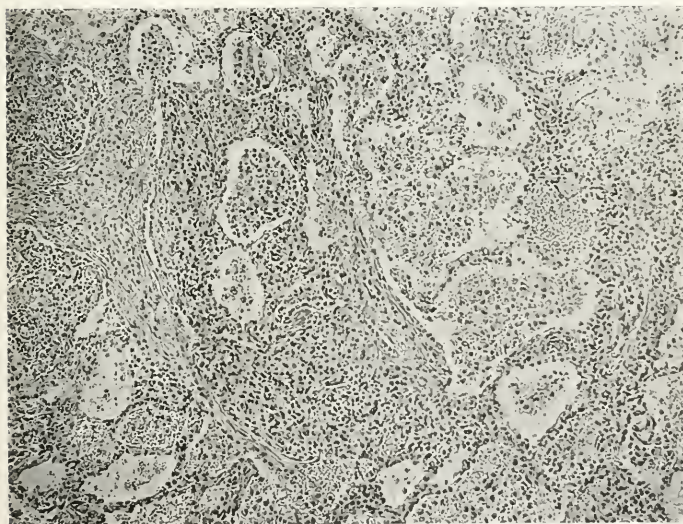


FIG. 31.

(Blake and Cecil: Experimental pneumonia. II.)

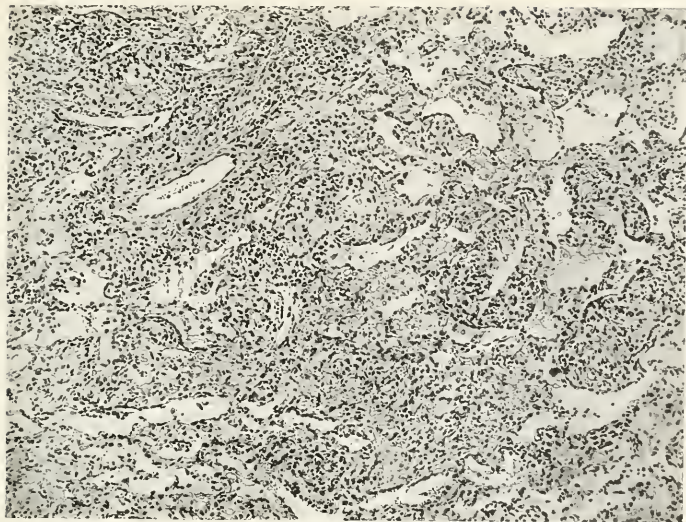


FIG. 32.

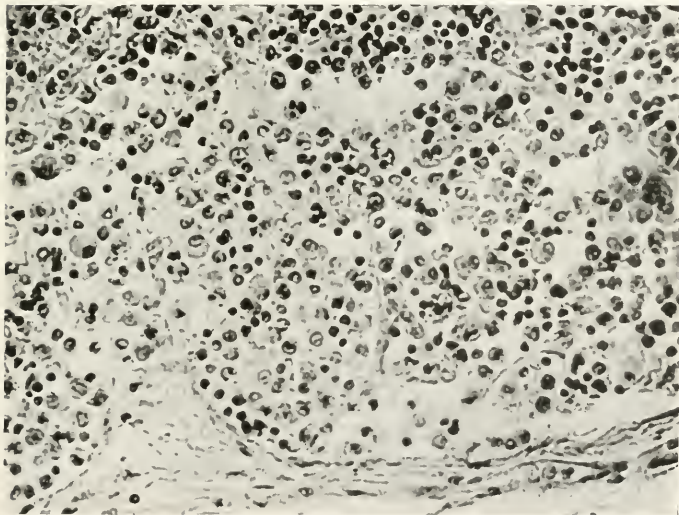


FIG. 33.

(Blake and Cecil: Experimental pneumonia II.)

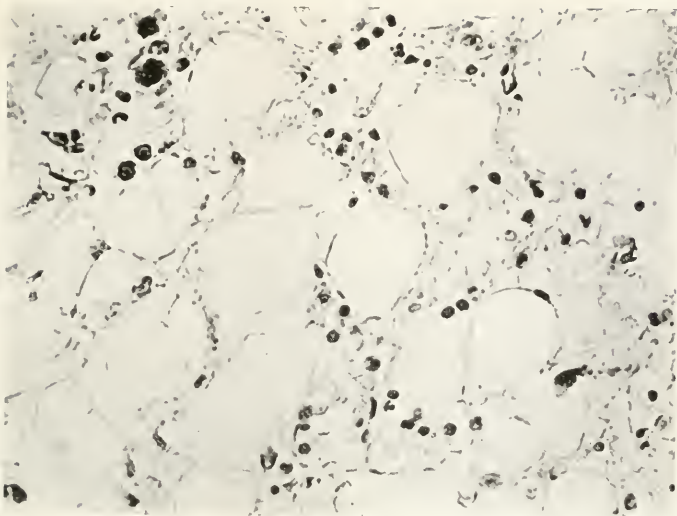


FIG. 34.

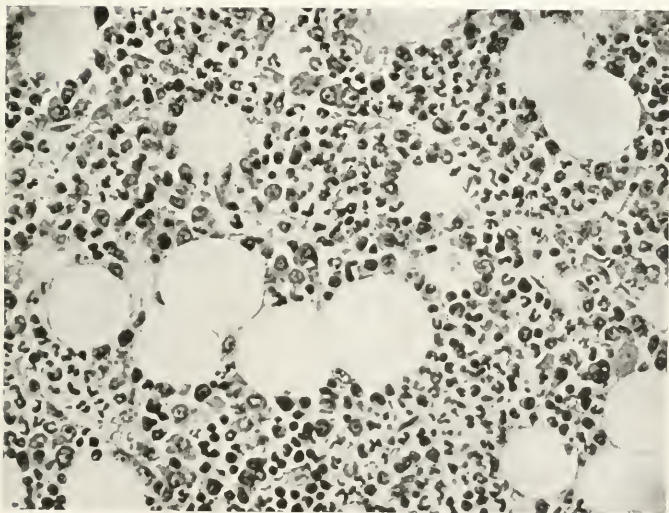


FIG. 35.

(Blake and Cecil: Experimental pneumonia. II.)

EXPERIMENTAL SYPHILIS IN THE RABBIT.

I. PRIMARY INFECTION IN THE TESTICLE.

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PLATES 45 TO 52.

(Received for publication, January 29, 1920.)

The successful transmission of the virus of human syphilis to rabbits in 1906 (1) gave promise of an unusual opportunity for the investigation of problems relating to syphilitic infection by the use of experimental means, and during the years following, a great deal of time was devoted to the study of the animal infection with a view to adapting it to this purpose. Numerous methods of inoculation were devised and perfected and the resulting infections were studied in great detail, but the hope of obtaining an experimental infection by local inoculation which was closely analogous to the human disease was not fully realized. Isolated instances of generalized infection with the occurrence of lesions of various types have been reported from time to time, and while some investigators have obtained such evidence of generalization in as many as 50 per cent of the infected animals (2), these occurrences have been comparatively rare in the experience of most observers.

It should be remembered, however, that much of the work which has been reported was based largely upon the study of animals inoculated with virus recently isolated from human sources or with virus which had been carried in rabbits for only a few years, and that while most investigators are of the opinion that the virulence of *Treponema pallidum* for rabbits may be increased by passage or by adaptation, it is still uncertain how long this increase in virulence can be kept up or to what extent it may be carried.

The infection in the rabbit, as in man, is one which runs a prolonged course and is very variable in character, so that it would

necessarily require years of observation and comparison of infections in large series of animals to reach any definite conclusion upon these points. Thus far, it is doubtful whether any one has been in a position to carry out such experiments upon a scale which would enable him to say what form of disease might ultimately be established in the experimental animal or how closely this disease might be made to resemble the human infection. From the standpoint of the use of the experimental infection as a means of investigating problems of human syphilis, it is obvious, however, that the animal infection should be brought to a stage of development as near that of the human infection as possible. From our own investigations it appears quite likely that this condition is much more nearly attainable than results thus far reported might indicate.

Among the problems of human syphilis which have been attacked through the use of the experimental infection in the rabbit, that of therapy stands out as of foremost importance, and our interest in experimental syphilis grew out of the use of the rabbit infection as a means of studying the therapeutic action of drugs. While at no time have we been able to undertake the study of the experimental disease as an independent problem, we have, nevertheless, been forced to carry out a great deal of collateral investigation as the need for information of a given character arose. In addition, we have had the opportunity of studying a large series of animals infected with two strains of *Treponema pallidum* both of which have been carried in rabbits for a number of years.

What we have to report at present, therefore, is not the result of a series of experiments designed expressly for the solution of particular problems in experimental syphilis, but the results of a series of observations supplemented by experiments intended to give information of a particular character.

The chief needs for therapeutic work upon experimental syphilis are reliable means of propagating the infection and an accurate knowledge of the disease as it exists in the experimental animal. It is the purpose of these papers, therefore, to condense the results of 4 years observation into a series of brief reports dealing with clinical aspects of experimental syphilis in the rabbit with especial reference to the phases of the subject which are of importance in carrying out

therapeutic experiments. Detailed discussion of individual papers will be deferred until the series has been completed, when the various aspects of the subject will be considered together. Later, we hope to be able to take up other phases of experimental syphilis which cannot be included in the present group of papers.

It will be found that our description of the experimental infection in the rabbit differs materially from others in the literature, but this is not to be taken as evidence of conflicting observations. We have dealt with older strains of *Treponema pallidum* than have commonly been used, and different principles have been employed in the handling of these organisms, so that it is not unlikely that the infection produced by us has been of a different order. Whether or not one uses such organisms to begin with, it is well to have in mind the state of virulence to which an organism may be developed and the character of the infection which may be produced by these organisms.

Primary Infections in the Rabbit.

Three general forms of local inoculation have been successfully employed in the rabbit. These include inoculation into the anterior chamber of the eye, inoculation into the testicle, and inoculation of skin surfaces—usually the scrotum. Of these, the first is at present chiefly of historical and scientific interest. Testicular inoculation was introduced by Parodi (3) in 1907 and soon became the most widely used form of inoculation. It is generally recognized that testicular infections are especially well adapted to the maintenance of pure strains of the organisms, but they are on the whole of less value as objects of clinical study, since a large part of the process takes place in parts hidden from direct observation, and the infection is of a less stable character than that produced by skin inoculations. There are, however, certain features of the syphilitic reaction which are brought out to greatest advantage by this form of infection, so that the consideration of the experimental disease may logically begin at this point.

EXPERIMENTAL.

Organisms Used.—Two strains of *Treponema pallidum* were used in the work to be reported. One was obtained from Dr. Hans Zinsser and Dr. J. G. Hopkins and was isolated by them from a mucous patch on November 17, 1913, being Strain A of their series (4). The other organism was the Nichols nervous strain V for which we are indebted to Colonel M. A. Reasoner, Medical Corps, U. S. Army (2).

The first of these organisms has been carried in rabbits in our laboratory for 4 years and had been isolated 2 years before it came to us. The Nichols strain was isolated from the spinal fluid of a case of neurorecidive in June, 1912, and has, therefore, been carried in rabbits for more than 7 years.

Animals Used.—During the course of the work, many types of rabbits were used. There were many animals whose markings indicated either a pure stock or a strong admixture of such breeds as the Belgian hare, the Flemish giant, the Dutch belt, the Himalayan, the silver-gray, the Polish, the New Zealand red, the Angora, the English, and perhaps others. The majority of the animals, however, were of the common varieties of albino, gray, brown, brindle, black, blue or maltese, and animals of mixed or broken coloring. We have used animals of all ages and degrees of testicular development, from the young rabbit of 3 or 4 months in which the testicles were still but slightly developed to the very old rabbit with redundant scrotum and atrophic testicles.

From records showing the character of each rabbit used, its weight, testicular development, character of the scrotum, etc., it was found that in as far as the testicular infection alone was concerned, almost any type of animal with well developed testicles would give good results, but that if other features of the infection were to be considered also, the best results were obtained from the use of the small or medium sized albino and common gray or brown rabbits. As far as possible, animals were selected in which the testicles were well developed, with a preference for young rather than old animals.

Technique of Inoculations.—Testicular inoculations were carried out by the use of a virus emulsion prepared from an infected testicle or skin nodule. The animal which served as a source for the virus was anesthetized and the testicle or nodule excised with aseptic precautions. This material, or a portion of it, was then placed in a sterile mortar and finely minced with scissors, after which enough sterile salt solution (0.85 per cent) was added to moisten the entire mass. The material was then rubbed to a thick paste, more salt solution being added and thoroughly mixed with the contents of the mortar; the amount of salt solution to be added was determined by the dilution of the spirochetes in the emulsion as indicated by dark-field examination. The emulsion ordinarily used contained not more than two to three spirochetes to a microscopic field nor fewer than a single organism to two to three fields. Such an emulsion was usually obtained with from five to ten volumes of salt solution to one of tissue. The only object in attempting to control the strength of the emulsion was to insure

the presence of a sufficient number of organisms to produce a prompt reaction and to maintain some degree of uniformity in the dose of virus used in successive transfers.

The fluid emulsion was aspirated into a small glass syringe fitted with a 22 gauge needle $\frac{3}{8}$ inch in length. The scrotum of the animal to be inoculated was sponged with 50 per cent alcohol, more for cleanliness than for antiseptic purposes, and 0.2 to 0.5 cc. of the emulsion was injected into the center of each testicle. The exact dose used was determined by both the strength of the emulsion and the size of the testicle to be inoculated. The precaution was taken to see that the needles used were sharp and smooth to avoid laceration and that the testicle was not unduly distended by the fluid injected.

This technique is one intended for intensive inoculations and never failed to produce a take. From time to time the procedure described was modified in several respects for different purposes, but we found it very simple of execution and entirely satisfactory for routine inoculations.

Material Studied.—From November, 1915, to September, 1919, 83 transfers of the two strains of *Treponema pallidum* were made by testicular inoculation. All the inoculated animals became infected and developed characteristic testicular lesions. Some were used for subinoculations and others for therapeutic experiments, but the course of the local infection was followed in a large number of animals from the time of inoculation to the spontaneous disappearance of the local lesions, and some animals were kept under constant observation for a much longer period of time.

Local Reaction to the Infection.

The infections produced in the testicles of rabbits inoculated with *Treponema pallidum* differed in many important respects. There were differences in the time and mode of onset and in the character and progress of the reaction as well as in the nature of the lesions developed, but on the whole the course of these infections tended to conform to a given plan and the variations were largely those of detail. The characteristic feature of the reaction observed in this series of animals was a tendency to the occurrence of a succession of changes separable into periods or phases of progression and quiescence or regression which gave to the local reaction a cyclic or relapsing course not unlike that which characterizes the infections produced by the blood spirochetes. This type of reaction occurred not only in the testicles but was the fundamental pattern of every phase of the syphilitic reaction in the rabbit, and forms, therefore, a logical basis for a consideration of syphilitic infections.

Incubation Period.

The time elapsing between inoculation and the development of testicular changes which could be recognized by external examination varied from 2 to 6 weeks, with occasional instances of longer or shorter periods of incubation. In fully 90 per cent of the animals, however, the incubation period fell within the narrower limits of from 3 to 4 weeks, and under properly chosen conditions of virus and rate of transfer, it was found that this time could always be brought within the 3 weeks period.

By dark-field examination of fluid obtained from the testicles, spirochetes were frequently demonstrated at a period well in advance of the appearance of clinical signs of infection. The same was found to be true of histological changes and not infrequently even gross alterations could be made out in the excised organ before any clinical sign of infection had become apparent. The time at which spirochetes and histological changes became demonstrable need not be reported in detail but was found to be within 7 to 10 days after inoculation.

Successive transfers did not necessarily shorten the incubation period to any appreciable extent. The dose of virus used, other things being equal, had a noticeable effect upon the speed of reaction, but this was not always the case. The vitality, or what may be termed the infectivity of the spirochetes, proved to be a factor of much greater importance than that of dosage. Thus, transfers made from actively developing lesions or from animal to animal as rapidly as the infection developed tended to produce or maintain a short incubation period, while inoculations made from old, inactive, or regressing lesions showed a relative prolongation of the incubation period irrespective of the dose of spirochetes used.

Mode of Onset.

The specific reaction in the testicle began in one of two ways, either as a small circumscribed focus of induration situated at the point of inoculation or as a diffuse swelling with increased tension of the entire testicle. When inoculations were made from old or inactive lesions or at relatively long intervals, the first form of reaction usually prevailed, but when transfers were made from one animal to

another in rapid succession, the reaction was more often of the second type. These differences in the character of the initial reaction were only temporary, as a rule, and tended towards a common type as the infection advanced.

Course of the Reaction.

Once the presence of a specific reaction became established, the progress of the infection was marked by certain changes such as enlargement and induration of the testicles which were common features of the reaction in all animals. The details of these changes necessarily varied in individual animals or even in the two testicles of the same animal, so that in general two extreme types of reaction with numerous modifications and variations became recognizable, depending upon the speed and sharpness with which successive changes occurred and the extent to which the several processes participated in the reaction.

In its highest form, the specific reaction in the testicle was characterized by a short incubation period, a diffuse onset and rapid development followed by a sharp crisis during which there was marked regression of the testicular lesions, a period of quiescence or inactivity, and one or more secondary cycles of active proliferation and quiescence or regression. Naturally, all these phases of the testicular reactions were rarely observed in their ideal form in one animal, but the several features of the reaction and some of its more common modifications may be illustrated by concrete examples.

Acute Exudative or Fulminating Reaction.—The first form of reaction to be cited is one which was characterized by an intense cycle of acute reaction terminating in crisis and followed by a slight secondary cycle of proliferative reaction. The case of orchitis shown in Figs. 1 to 6 furnishes an example of a reaction of this kind.

Fig. 1 shows the testicles at the time of inoculation. 15 days after inoculation, there was an increased tension and slight swelling of the testicles which progressed rapidly for about 6 days, the left testicle being more affected than the right (Figs. 1 to 3). At first, the scrotum was drawn tightly about the testicle, its vessels were slightly prominent, and there was a faint reddening of the skin surface (Fig. 2). On the 6th day, the reaction reached its height (Fig. 3), having

culminated in a diffuse congestion and marked edema of the scrotum most of which had developed within the last 24 hours.¹

On the 7th day after the appearance of the specific reaction, there was a sharp change during which the congestion and edema of the scrotum subsided very rapidly and were followed somewhat more slowly by a decrease in the swelling of the testicle and a softening of the induration. Within 3 days, regression had reached the point indicated in Fig. 4. At the end of a week, the testicles had returned to approximately normal size and appeared as small, rather soft, atrophic masses with an area of thickening at the lower pole of the right testicle.

No further change was noted during the succeeding 10 days. Then on the 12th day, the mass at the lower end of the right testicle was found to be definitely enlarged and indurated. This marked the beginning of a second cycle of progressive reaction which at this time was limited to a circumscribed area in one testicle. 7 days later, the nodule in the right testicle had increased to nearly 1 cm. in diameter and a few small shotty nodules were palpable in the left testicle (Fig. 5).

The lesion on the right continued to develop for another week at which time it presented the appearance shown in Fig. 6. Meantime, the nodules on the left had disappeared completely and there was no further reaction in this testicle. During the next 2 weeks, the nodule on the right showed several short periods of quiescence and slight activity and then underwent complete resolution.

The difference in the intensity and extent of the reaction in the two testicles of this animal is of especial importance. It will be seen by comparing the photographs that the acute reaction in the left testicle was much more intense than that in the right, and that when the crisis came, regression in this testicle took place more rapidly and was more complete. In like manner, the secondary or proliferative reaction, while slight in both testicles, was decidedly less in the left than in the right. This relation between the intensity of the first cycle of reaction on the one hand, and the secondary reaction on the other was so constant as almost to establish a rule of inverse proportions between the first and second cycles of reaction in a given animal.

¹ At this stage of the infection, the scrotum and scrotal sac were filled with a gelatinous exudate or with a clear straw-colored or slightly blood-stained fluid which coagulated quickly on standing. The testicles also contained a fluid exudate of similar character which dripped freely when the organ was cut. Microscopic examination of these structures showed an inflammatory exudate composed chiefly of serum and fibrin with some polymorphonuclear leucocytes and red blood cells.

Chronic or Proliferative Reaction.—The second general type of reaction which occurred in the testicles of infected rabbits was one in which infiltration and acute exudative phenomena were subordinated to processes of proliferation. Reactions of this type showed, therefore, a more gradual onset and development of lesions with less marked cyclic alterations. In this group, the infection frequently began as a circumscribed focus of induration which gradually spread until the entire testicle became involved. Enlargement of the testicle took place more slowly as a rule than in the preceding group of cases and was associated with induration rather than increased tension of the testicle. The infection usually progressed steadily for upwards of 2 to 3 weeks by which time the testicle presented the appearance of a large, smooth or slightly nodular organ of extreme hardness. Congestion of the scrotum was comparatively slight, and edema, when present, was less marked than in the typically acute cases.

Instead of a pronounced crisis, progress of the reaction ceased more gradually and was followed by a period of inaction or of comparatively slow regression during which the testicles diminished in size and softened to a greater or less extent. If edema was present, it disappeared more rapidly, but at the end of the period of regression, the testicle still remained definitely enlarged and indurated. This residual induration was sometimes diffuse or uniform in distribution, while at others it was limited to certain areas or portions of the testicle.

Growth of the lesions was then resumed and continued with occasional remissions so that the progressive tendency of the reaction remained uppermost for a considerable period of time. The reaction in the testicles then subsided and the lesions resolved in much the same way as they had developed.

Two examples of this type of reaction are given in Figs. 7 to 9 and 10 to 12 which represent stages in the progress of the reaction in two animals of the same series. The incubation period in these two animals was 29 and 22 days respectively. In the first animal (Figs. 7 to 9), there were diffuse enlargement and induration which were first noted about 4 weeks after inoculation; the infection involved the scrotum as well as the testicles proper. The reaction reached its height in the left testicle 36 days after inoculation (Fig. 7) and in the right 39

days (Fig. 8). Both testicles then showed a slight regression (indicated in the photographs by a slight decrease in size) followed by renewed activity toward the end of the 7th week after inoculation (Fig. 9).

The reaction exhibited by the second animal differed somewhat from that of the first. There were again a diffuse enlargement and induration of both testicles, but, as indicated by a shorter incubation period, the reaction took place more rapidly, reaching its height during the middle of the 4th week after inoculation (Fig. 10). At the time the reaction was nearing its height in the first animal, a decided regression had already taken place in the second (Fig. 11). This regression was more marked than in the first case, and a well marked second cycle of reaction was not apparent until towards the end of the 7th week. The character of this reaction is indicated in Fig. 12 which shows the condition present 8 weeks after inoculation.

Variations in the Specific Reaction. *Subacute Reactions.*

The modifications and combinations of these two fundamental types of reaction were, as we have said, quite numerous, but a few specific examples will serve to indicate the character and direction of the more important variations.

The first variation which may be mentioned is one concerning the acute type of reaction. The photographs reproduced in Figs. 13 to 15 illustrate a reaction which was characterized by an acute cycle of moderate extent associated with some diffuse induration in the testicle which persisted after the crisis. The reaction began in this animal as a circumscribed focus of induration in the posterior portion of the testicle, which was first detected 18 days after inoculation. Induration spread and the testicle enlarged rather rapidly, at the same time becoming diffusely indurated. The height of this change was reached 26 days after inoculation (Fig. 13). There was a moderate edema of the scrotum which lasted for a few days and then subsided, leaving the testicle still slightly enlarged as indicated in Fig. 14. There was some further regression of the lesions, and renewed activity did not set in until towards the end of the 7th week. This second cycle of reaction was both focal and diffuse in character and at the time the third photograph of the series was taken (Fig. 15) there were diffuse induration of both testicles and numerous nodules varying from a few mm. to approximately 0.5 cm. in diameter.

A second type of orchitis which was quite common in our series of animals is shown in Figs. 16 to 18. The onset of the infection in this instance was again of a circumscribed nodular character with an incubation period of 27 days. The testicles became diffusely indurated and showed a marked enlargement. This reaction took place somewhat more slowly than in the preceding case and did not

reach its full development until about 6 weeks after inoculation (Fig. 16). At the time the first photograph was taken (Fig. 16) the left testicle was retracted within the abdominal cavity and could not be brought through the inguinal canal, and there was very marked edema of both scrotal sacs which is best shown on the right. Edema subsided rather slowly, and at the same time the testicles diminished somewhat in size and became slightly softened. On the 49th day (Fig. 17) the scrotum showed a diffuse thickening with beginning induration over the ventral surface of both testicles; the testicles themselves were still considerably enlarged and markedly indurated. From this point onward, the reaction gradually shifted from the testicles proper to the scrotum with the production of chancre-like lesions, the beginning of which is indicated in Fig. 18.

The feature of especial interest in the reaction exhibited by this animal was the slowly progressive but pronounced character of the reaction, culminating in a diffuse swelling and edema of both the testicles and the scrotum and the subsequent transference of the center of reaction from the testicles to the scrotum—the latter condition being a frequent occurrence in testicular infections.

Another group of photographs illustrating an infection of somewhat the same character is reproduced in Figs. 19 to 22. This animal was an old albino with rather atrophic testicles and, as is usually the case with such animals, the reaction was slow to develop. Infection began as a circumscribed focus of induration which was recognized about 4 weeks after inoculation and gradually spread until the entire testicle was diffusely involved. There was a slow but steady increase in the size and induration of the testicles, extending over a period of about 2 weeks. The height of the reaction in the right testicle was reached 46 days after inoculation (Fig. 19) and a few days later in the left. There were moderate congestion and edema of the scrotum which lasted for several days. Crisis occurred, followed by regression, but at the end of this phase of the reaction, both testicles were still diffusely enlarged and indurated much as they appear in Fig. 20. Within 10 days after the crisis, there was renewed growth of lesions situated in the tail of the epididymis (Fig. 21). These lesions grew actively for another 2 weeks at which time they formed large indurated masses of irregular shape, involving the skin as well as the epididymis (Fig. 22). In the meantime, the reaction in the remaining portions of the testicle had completely subsided, leaving the testicles as small atrophic masses.

A fourth example of a more unusual reaction is given in Figs. 23 to 25. The earlier stages of the reaction in this animal were of an ordinary chronic proliferative type and had progressed to the point of the formation of a scrotal chancre on the left associated with induration of the testicle itself with marked enlargement and induration of the right testicle and slight extension to the scrotum at its lower pole, when, towards the end of the 7th week, there was an acute exudative reaction in the right testicle and scrotum, which reached its height on the 53rd day after inoculation. This reaction was followed by a typical crisis (Figs. 23 and 24), and both testicles then began to diminish in size and induration,

while the lesions of the scrotum continued to grow actively (Fig. 25). The feature of especial interest here was the occurrence of an acute exudative reaction late in the course of infection.

This small group of cases, including the more acute and chronic forms of reaction together with various modifications and combinations of these two fundamental types of processes, is typical of the reactions seen in practically all cases of outspoken testicular infection. Many of these modified reactions might be spoken of as subacute in the same sense in which the others are acute or chronic in character, since they combine to a greater or less degree the features of exudation and infiltration with those of proliferation of fixed tissue cells. It will be noted, however, that whatever the variation in the response of the individual animal, they all show an unmistakable tendency towards a reaction of a recurrent or a relapsing type.

It was found that these reactions could be influenced to a considerable extent in several ways. Thus, reactions of the more acute type were especially frequent when transfers were made from one animal to another in rapid succession or during the ascending phase of the acute reaction. In a small proportion of animals, the local infection terminated with a single cycle of reaction such as that described. As a rule, however, the acute cycle of reaction was followed by other changes leading to the formation of lesions differing in many ways from those originally produced. The occurrence of these secondary cycles of reaction depended to a considerable extent upon the character and extent of the first cycle. Thus animals in which there was an intense, acute reaction were, as a class, less apt to show secondary reactions, of marked degree than those in which the process had been more gradual or less intense.

Reactions tending towards the chronic or proliferative type were quite common and occurred with especial frequency after inoculations made at long intervals or from chronic indurative lesions. They were frequently associated with the most persistent local infections and gave rise to some of the most conspicuous and destructive lesions of the testicles.

Late Developments of the Testicular Reaction and the Character of the Lesions Produced.

Most writers have treated the testicular infection of the rabbit from the standpoint of the lesions produced, but we prefer to regard these lesions more as manifestations of a reaction to infection and hence very little has been said concerning the lesions themselves. Following out their ideas of the importance of the lesion as an entity in itself, many investigators have attempted to differentiate between the various types of lesions produced, and several classifications have been proposed, based largely upon the location of the lesion and the character of the pathological process (5, 6). In our experience such differentiations would be very difficult to make except as applied to a particular lesion at a particular time or to what may be termed the residual lesions which ultimately come to be established.

Without entering into a detailed discussion of this phase of the subject, it may be said that while the infection in the animals studied by us began either as a circumscribed or as a diffuse process, neither of these conditions was permanent, and the same applied to the character of the pathological process. In practically all instances, the infection ultimately involved the entire organ with the production of lesions in the parenchyma, tunics, epididymis, and cord, and in many instances in the scrotum as well. This extension took place early, as a rule, and by the time the reaction had reached its height, there was what might be termed a panorchitis.

In many animals, this widespread involvement was of a perfectly uniform character as far as could be determined by the gross appearance of the organ or by palpation. In a second group of animals, there was a finely granular condition of the testicle, while in a third, distinct indurated nodules could be recognized, separated by tissue showing a lesser degree of involvement or an involvement of a different character. This differentiation into lesions of a particular type or lesions situated in certain localities came as a late development in the course of the infection and was, in our opinion, attributable to the character of the reaction against the local infection and the tendency on the part of the infection to extend in certain directions. During the later stages of the infection, therefore, one fre-

quently had to deal not with a widespread infection at the height of its activity but with an infection which was localized only in certain areas. The points of most frequent involvement were the globus minor and major, the tunics, the mediastinum testis, and the skin, or, in other words, the areas in which membranes or connective tissue in some form was most abundant.

These later developments and extensions of the testicular infections are of considerable interest both from the standpoint of the reaction to infection and the lesions which are produced. Several examples of this phase of the local reaction have already been given in the preceding illustrations, which may be supplemented by Figs. 26 to 37.

The first group of photographs in this series (Figs. 26 to 28) illustrates an infection which began as a diffuse indurative orchitis. At the period of infection represented in Fig. 26, 58 days after inoculation, a more or less diffuse extension of the infection to the scrotum of the right testicle had taken place, while on the left, the lesions in the testicle and scrotum were assuming a more nodular character, and these differences in the character of the changes in the two testicles persisted to the end. As the infection advanced, a large portion of the right testicle and scrotum underwent diffuse necrosis; on the left, the lesions began to be more circumscribed and two well defined scrotal lesions were formed (Fig. 27). Eventually, a large portion of both testicles underwent necrosis as indicated in Fig. 28, which shows the condition 80 days after inoculation. Even at this stage, however, the multinodular character of the lesions of the left testicle could still be made out, there being three fairly well defined centers of reaction.

In this animal, the feature of especial importance in the reaction was the tendency to widespread necrosis which eventually involved the entire skin surface and the testicle as well. This was not always the case, however, even when the reaction in the testicle was fully as marked as in this instance. In Fig. 29, a case of orchitis is illustrated in which the involvement of the testicle was also quite marked, but the necrosis and ulceration were confined to two more or less circumscribed areas producing effects more analogous to primary skin lesions. Lesions of this type were very common among our animals, and all gradations and transitions could be found between the lesion which was definitely a diffuse orchitis with necrosis and ulceration and lesions which were identical in character with primary skin lesions. The photographs reproduced in Figs. 30 to 32 are given to illustrate this point.

Fig. 30 shows a condition which is clearly an orchitis with skin involvement which has led to the formation of depressed ulcers with a definitely indurated collar such as is seen in primary skin lesions. In Fig. 31, taken only 6 days later, the picture has changed somewhat, and the whole mass in the left testicle

has filled out, while the right testicle now shows a single large ulcerated lesion identical with the large skin chancres which develop in the rabbit.

The next step in the series of transition is shown in Fig. 32. The infection in this animal began as a diffuse indurative orchitis which later became nodular with lesions such as those shown in Fig. 21, except that there was an active nodule situated in the tunic of the left testicle. The nodule in the epididymis of the right testicle extended to the skin, forming a chancre-like mass seen in Fig. 32. On the left, the nodule in the tunic was the one to develop most actively, forming a skin lesion still more like the ordinary chancre. These lesions healed in the course of about 4 weeks, leaving an active nodule in the epididymis of the left testicle (Figs. 33 and 34).

A final transition in the course of the testicular infection may be illustrated by Figs. 35 to 37. The infection in this animal began as a circumscribed focus of induration which quickly developed into a diffuse orchitis (Fig. 35). In time, the diffuse induration resolved, leaving small focal lesions in the tail of the epididymis of both testicles. Foci of infection then appeared in the skin and developed into the lesions shown in Fig. 36 (77 days after inoculation). Upon removal of the testicles and skin lesions, the testicles were found to be small atrophic masses entirely free from any gross evidences of active infection (Fig. 37). On the right, there was an indurated lesion in the epididymis which in part was continuous with the skin lesion; on the left, the two processes showed less connection and one of the two lesions in the scrotum was entirely distinct from the other lesions present (Figs. 36 and 37).

In this animal an infection which began as a circumscribed focus of induration first became diffuse, was then transformed into a nodular epididymitis, and the seat of active infection was finally transferred to the scrotum, leaving the body of the testicle free from active lesions.

Atypical and Low Grade Reactions.

Before leaving the subject of the local reaction in the testicle, mention should be made of another class of infections. There were, in our series, a few animals which showed some peculiarity in their response to infection, such as the development of slight or otherwise atypical lesions. These infections may be spoken of as atypical, on account of such peculiarities in the reaction, or as low grade reactions, in the sense that the reaction lagged or that the lesions which developed were of a minor character.

The common instances of this kind were slight reactions of short duration and slight but persistent reactions. Of the latter class, there were two main groups of infections, one in which a diffuse or

circumscribed focus of reaction developed very slowly and never resulted in more than a slight diffuse thickening in the testicle or a small nodular induration. The second group included instances in which a slight diffuse reaction subsided with the formation of residual nodules or in which an initial focus of infection was later supplemented by the appearance of secondary foci of a similar character. Minor lesions such as these not infrequently persisted for months without showing any especial sign of activity or growth.

Most of the infections of this class occurred during the earlier part of the work and have rarely been seen during the last 2 years. We are inclined to attribute occurrences of this kind to the circumstances under which the transfers were made.

From our present knowledge of conditions which influence the specific infection in the rabbit, we recognize this class of cases as attributable to certain definite causes, mainly to an attempt to inoculate with spirochetes at a time when their vitality, or infectivity, had been materially reduced by cyclic immunological reactions (page 492), but also to peculiarities in the response of individual animals to the specific infection.

Accessory Skin Lesions.

For the sake of completeness, mention may be made also of the occurrence of accessory lesions in the scrotum which develop along the path of the needle as a direct result of the process of inoculation (Figs. 2 and 4). At times, these lesions appeared before any reaction in the testicle had become recognizable. Without going into a discussion of this feature of testicular infections, which properly belongs in the section on scrotal chancres, it may be said that these foci of infection appeared in one of three forms, as small gelatinous swellings in the skin, as translucent pearly nodules in the superficial layers of the skin, or as opaque points or plaques in the depths of the scrotum, usually upon the outer surface of the tunica vaginalis. They rarely developed to lesions of any considerable extent. In some instances, however, they did develop into large lesions either as independent foci of reaction or as indistinguishable parts of the testicular reaction.

The Spirochete Reaction and the Spirochete Content of Lesions.

The above observations upon the course of the specific reaction in the testicle were supplemented by parallel dark-field examinations of fluid taken from the testicular lesions at different periods of the infection in order to determine what changes, if any, could be demonstrated among the infecting organisms while the changes in the testicles were in progress. From these examinations, it was found that as the infection progressed, the spirochetes exhibited changes analogous in character to those described in the testicles themselves. As we have already mentioned, spirochetes began to multiply and were present in considerable numbers before any gross manifestation of infection had become recognizable. This increase in spirochetes continued parallel with the development of the lesions, so that by the time the lesions had reached the height of their first cycle of development, actively motile spirochetes were present in large numbers.

At this point, the spirochetes suddenly began to lose their motility and to collect in tangled masses. In cases of acute orchitis, these changes coincided roughly with the development of edema of the scrotum and were followed promptly by regression of the testicular lesions.

Following the phenomenon of agglomeration, the spirochetes rapidly diminished in numbers, so that within a few days, organisms were difficult to find in fluid aspirated from the testicles, and those seen were either degenerated or showed but slight signs of motility; in many instances no organisms could be found.

After passing through a crisis such as this, actively motile spirochetes again appeared in the testicular fluid, and increased in numbers, presaging a renewed activity on the part of the lesions or at least a cessation of regression for the time being. These parallel changes continued throughout the existence of the local infection—the change in spirochetes usually occurring slightly in advance of the changes in the lesions.

As in the case of the lesions, cyclic changes were at times very sharp and easily recognizable, while at others they were less marked and might readily have escaped detection had it not been for the example of the sharper type of reaction.

During the latter part of the infection, the spirochetal content of lesions was found to be more difficult of estimation and somewhat uncertain. Where a single lesion was present, diffuse or circumscribed, the spirochetes in one area might diminish or disappear while they were present in considerable numbers or were actively increasing in other areas. These changes in the spirochetal content had their parallel, however, in the shifting centers of growth in the lesions themselves or, in the case of multiple lesions, in the resolution of one lesion while another was undergoing active development. By careful study of the lesions, it was found, however, that actively growing lesions or portions of lesions always contained actively motile spirochetes, and the same was true of many lesions which were merely quiescent but not regressing. The spirochetal content of lesions which were regressing was more variable; in many instances, spirochetes could not be detected by dark-field examination, while in others, they could still be demonstrated in fair numbers which diminished as the lesions resolved.

The changes affecting the spirochetes within the lesions will be recognized as entirely analogous to those which occur in blood stream infections with such organisms as *Spirocheta recurrentis*. In order to assure ourselves that the apparent immobilization, agglomeration, and degeneration described, entailed some actual alteration in the pathogenic properties of these organisms, tests were carried out by animal inoculation. For this purpose, inoculations were made with organisms taken as nearly as possible at the height of the first cycle in cases of intense, acute reaction, or more properly at the beginning of the crisis, and the tests were controlled by a parallel series of inoculations made with actively motile spirochetes taken from an early stage of testicular infection.

From these tests it was found that the infecting power of such organisms was markedly diminished. In one experiment, a series of animals inoculated with approximately ten times the dose of immobilized and agglomerated organisms that was used in the controls showed an incubation period of 6 weeks as contrasted with 3 weeks in the controls, while the lesions were slow to develop and were less pronounced than in the control animals. Similar observations as to the infectivity of the spirochetes at different periods of the infection have

been made many times and there is no doubt in our minds as to the significance of the cyclic reactions described.²

Duration of the Local Infection.

The duration of the local infection as determined by the presence of active lesions was as variable as the course of the infection itself, and no fixed limits can be given either for the several phases of the local reaction or for the infection as a whole. The period of active infection varied anywhere from 1 to more than 12 months. In some animals, the entire reaction was represented by one intense cycle of acute reaction which terminated within 4 to 6 weeks after inoculation; in others, the infection continued through successive cycles of reaction, but the period of active infection was rarely longer than 2 to 4 months. Inactive or latent lesions which showed occasional periods of slight activity frequently persisted much longer, and residual lesions in the epididymis, skin, and tunics not infrequently persisted for from 4 to 8 months. In general, the duration of the local infection was inversely proportional to the intensity of the local reaction.

CONCLUSIONS.

The conclusions which might be drawn from this series of observations are very numerous, but we shall refer briefly to only a few of the more important points.

From the standpoint of a pathological process, it is important to note that the local response is not altogether a granulomatous reaction. In fact, it appears that exudation and infiltration are the fundamental processes and that proliferation is a secondary phenomenon.

In the second place, it is quite clear that the reaction to infection in the testicle, and hence the course of the infection itself, are of a periodic or relapsing character, analogous in this respect to other spirochete infections, or for that matter present certain protozoan

² We have evidence sufficient to indicate that rabbits inoculated in the testicles or scrotum with *Treponema pallidum* always show a blood stream invasion, and that these organisms are subject to changes analogous to those which occur in the testicles. The details of this work will be reported later.

characteristics. To what extent this infection is influenced by a local reaction and to what extent by a systemic reaction are at present unknown, but as far as the local infection is concerned, it appears to be subject more to local than to general conditions. Further than this, it appears that the character of the reaction which takes place in a given case is itself significant. The relation between the reaction in the individual animal and the duration of the infection represents a constant. When the local reaction assumes the character of a chronic proliferative process, the life of the infection is prolonged and the extent of the reaction which takes place before the infection is brought under control is proportionately increased; when, however, the reaction assumes the form of an intensely acute reaction, the life of the local infection is promptly terminated. This relation between the local reaction and the duration of an active infection, together with the determination of the character of the experimental infection in the rabbit, are perhaps the two most important deductions to be drawn from this series of observations in that they touch every phase of the experimental infection.

SUMMARY.

A study was made of the infections produced in rabbits inoculated in the testicles with two strains of *Treponema pallidum* which had been carried in rabbits for several years. Infection resulted in all instances; the incubation period varied as a rule between 2 and 6 weeks and under properly chosen conditions could be reduced to approximately 3 weeks or less.

The resulting infection pursued a typically cyclic or relapsing course which affected both the spirochetes and the associated lesions in the testicle. The spirochetes in the local lesions exhibited periodic changes less marked and less regular but identical in character with the changes which occur in the blood in cases of relapsing fever. The lesions in the testicle also showed periods of active development and quiescence or regression which followed closely upon the changes exhibited by the spirochetes.

The specific reaction in the testicle showed considerable variation in the speed and sharpness with which successive phenomena occurred

as well as in the character and extent of the processes themselves. These reactions were of two fundamental types. In one group of animals, the reaction was characterized by an intense cycle of acute exudation and infiltration with a lesser degree of proliferation, followed by crisis and subsequent recurrence of secondary cycles of proliferative reaction of a minor degree. In the other group of animals, the reaction was more chronic in character and consisted largely of infiltration and proliferation. The progress of the reaction was more gradual, and sharp alterations in its course were absent. The infection progressed by a succession of stages with slight and irregular remissions.

In a third group of animals, the reaction was subacute, combining at the same time the processes of exudation, infiltration, and proliferation. The first cycle of reaction was fairly acute and terminated in a definite crisis with moderate regression which in turn was followed by recurrence and more or less pronounced secondary cycles of proliferation.

In all cases of outspoken infection, there was diffuse involvement of testicle, tunic, epididymis, and cord, but as the infection progressed, the lesions underwent many transformations, so that a variety of lesions was formed from processes which in the beginning were of a common type. Eventually, the reaction became more irregular and the infection became centered in one or more foci which were commonly situated in the epididymis, tunics, scrotum, or mediastinum testis. These centers served as residual foci of infection.

The duration of the testicular process was found to be very variable. In some animals, the entire reaction consisted of but a single sharp cycle, and the local infection was terminated by crisis within 4 to 6 weeks after inoculation. As a rule, the period of active infection was from 2 to 4 months, and quiescent or inactive lesions not infrequently lasted for from 4 to 6 months. In exceptional instances, local infection persisted for more than a year.

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EXPLANATION OF PLATES.

The figures are reproductions of untouched photographs which represent the objects at their natural size. The statements of time refer in all instances to the time after inoculation, unless otherwise stated.

PLATE 45.

FIGS. 1 to 6. An acute diffuse reaction in the testicles and scrotum with crisis and regression followed by a slight secondary cycle of reaction and the formation of circumscribed nodular lesions.

FIG. 1. The normal testicles.

FIG. 2. 20 days. Well advanced, acute orchitis with beginning congestion and edema of the scrotum.

FIG. 3. 24 hours later. The height of the first cycle of reaction with intense congestion and edema most marked in the left testicle.

FIG. 4. 72 hours after the crisis. Note the greater regression of the left testicle.

FIG. 5. 48 days. The second cycle of reaction and the formation of nodular lesions in both testicles.

FIG. 6. 56 days. The lesion in the tail of the epididymis and scrotum of the right testicle is still increasing, while the lesions have disappeared from the left.

PLATE 46.

FIGS. 7 to 9. Chronic proliferative orchitis with scrotal involvement.

FIG. 7. 36 days. The height of the first cycle of reaction in the left testicle. Note the perfectly uniform enlargement of the testicle extending even to the cord. The scrotum of both testicles is involved, and on the right, there is an area of beginning necrosis.

FIG. 8. 39 days. The height of the first cycle of reaction in the right testicle; the crisis in the left has passed and the lesions are regressing.

FIG. 9. 51 days. The second cycle of reaction in progress in both testicles. Note the smooth, tense scrotum of the right testicle indicative of diffuse involvement, while in the left the lesions are now assuming a multinodular character.

FIGS. 10 to 12. Chronic proliferative orchitis in another animal of the same series as that in Figs. 7 to 9.

FIG. 10. 24 days. Well marked diffuse induration of both testicles approximately at the height of the first cycle of reaction.

FIG. 11. 36 days. Decided regression of the lesions of both testicles.

FIG. 12. 56 days. An early stage of the second cycle of reaction.

PLATE 47.

FIGS. 13 to 15. An acute diffuse reaction of moderate degree followed by crisis and a second cycle of chronic proliferation, diffuse and focal in character.

FIG. 13. 26 days. The height of the acute reaction with moderate edema of the scrotum. The left testicle is larger than the right.

FIG. 14. 28 days. Regression. The left testicle is still larger than the right due to a greater degree of proliferation in the initial reaction. Compare Figs. 13 and 14 with Figs. 3 and 4.

FIG. 15. 53 days. Moderate enlargement and induration of both testicles.

FIGS. 16 to 18. Subacute orchitis characterized by marked induration and edema of both testicles and scrotum and gradual shifting of the center of reaction from the testicles to the scrotum.

FIG. 16. 41 days. The left testicle is retracted within the abdominal cavity.

FIG. 17. 49 days. Some regression has taken place, but the scrotum is thickened and both testicles are still diffusely enlarged and indurated.

FIG. 18. 52 days. The lesions in the testicles are still slowly diminishing, but focal lesions are beginning to develop in the scrotum.

PLATE 48.

FIGS. 19 to 22. A subacute reaction with diffuse involvement of both testicles followed by a second cycle of reaction localized in the tail of the epididymis.

FIG. 19. 46 days. The height of the first cycle of reaction in the right testicle.

FIG. 20. 53 days. The extent of the regression following the crisis in the reaction is shown. Note that at the lower end of the right testicle, there is a nodule which has not regressed to the same extent as the rest of the testicle.

FIG. 21. 60 days. Continued regression of the testicular lesions with development of focal lesions in the epididymis.

FIG. 22. 76 days. Focal lesions of the epididymis and scrotum.

PLATE 49.

FIGS. 23 to 25. An acute exudative reaction occurring during a late stage of the local infection.

FIG. 23. 53 days. Chronic proliferative changes in both testicles with localized lesions of the scrotum with acute congestion and edema on the right.

FIG. 24. 2 days later. Regression on the right with slight swelling of the testicle and increasing growth of the scrotal lesion on the left.

FIG. 25. 61 days. Regression of the testicular lesions with simultaneous growth of the lesions in the scrotum of both testicles.

PLATE 50.

FIGS. 26 to 28. Chronic orchitis with extensive necrosis of testicles and scrotum. Right diffuse; left nodular.

FIG. 26. 58 days. Diffuse induration of the right testicle and scrotum with beginning skin necrosis and multinodular lesions of the left testicle.

FIG. 27. 73 days. Diffuse necrosis of the right testicle and scrotum; focal necrosis of the left.

FIG. 28. 80 days. Later stage of same lesions. Note lines of demarcation between the three masses on the left.

PLATE 51.

FIG. 29. Chronic diffuse orchitis with circumscribed lesions of the skin resulting from metastatic infection of the testicles.

FIG. 30. Chronic diffuse orchitis with multiple chancre-like lesions of the skin.

FIG. 31. Same testicles 6 days later showing transformation of the lesions. Note especially the single large chancre-like mass in the right testicle.

FIGS. 32 to 34. Late transformations in a case of testicular infection.

FIG. 32. Chancre-like extensions to the scrotum.

FIG. 33. Healing of the skin lesions with an active nodule persisting in the epididymis of the left testicle.

FIG. 34. 93 days later. Skin lesions practically healed, while the nodule in the epididymis is still active.

PLATE 52.

FIGS. 35 to 37. Diffuse orchitis with eventual transference of the local infection from the body of the testicles to the epididymis and scrotum.

FIG. 35. Diffuse indurative orchitis of both testicles.

FIG. 36. The final lesions in the epididymis and scrotum.

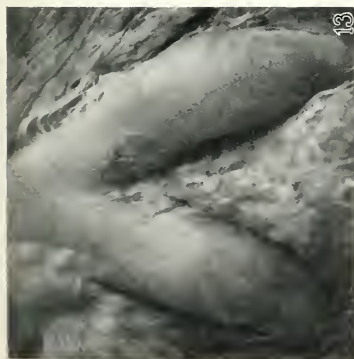
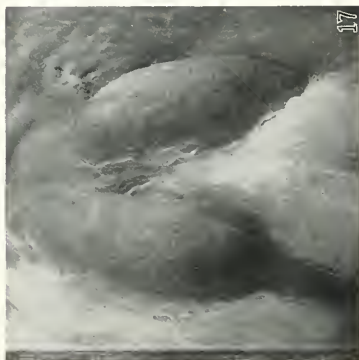
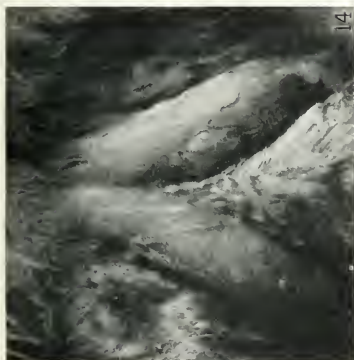
FIG. 37. The same lesions excised and sectioned. Both testicles are atrophic; on the right, the nodule in the tail of the epididymis fits into a hollow beneath the ulcer in the skin, but the two lesions are not entirely fused with each other; on the left, there is only a small nodule in the epididymis which is fairly distinct from the two lesions in the scrotum.

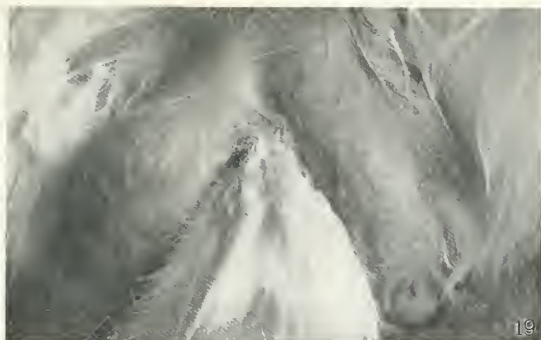


(Brown and Pearce: Experimental syphilis in the rabbit. I.)

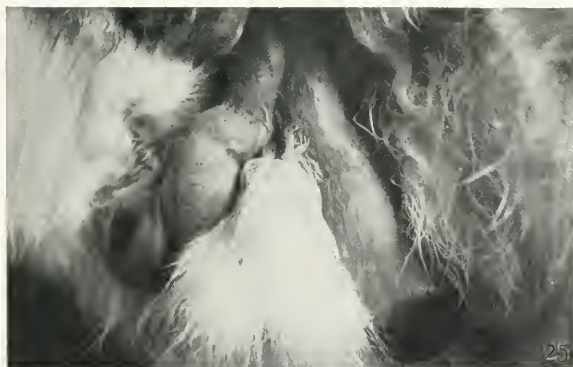
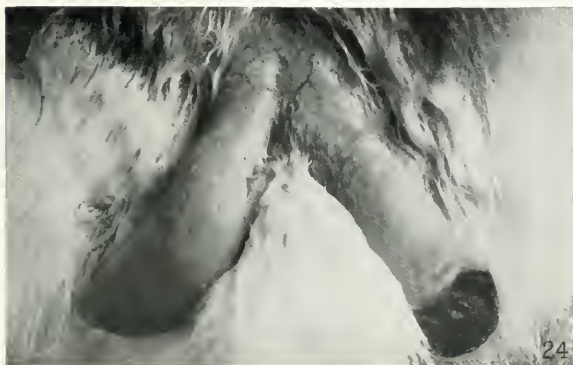


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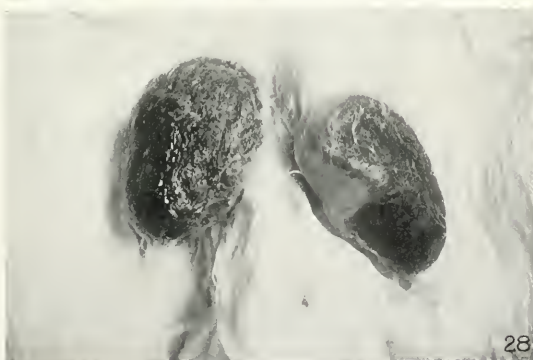




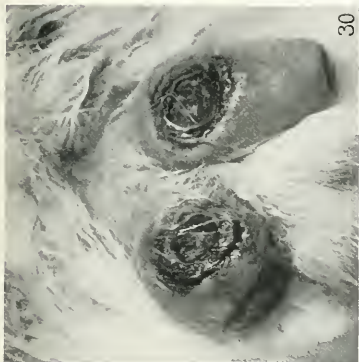
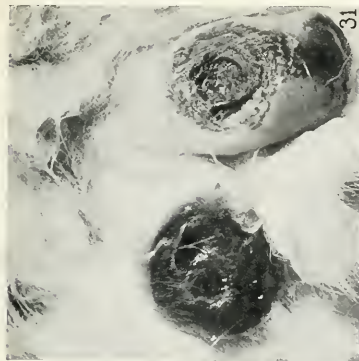
(Brown and Pearce; Experimental syphilis in the rabbit. I.)



(Brown and Pearce: Experimental syphilis in the rabbit. I.)



(Brown and Pearce: Experimental syphilis in the rabbit. I.)



(Brown and Pearce: Experimental syphilis in the rabbit. I.)



(Brown and Pearce: Experimental syphilis in the rabbit. 1.)

STUDIES ON EXPERIMENTAL PNEUMONIA.

III. SPONTANEOUS PNEUMONIA IN MONKEYS.

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PLATES 53 TO 64.

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During the course of the investigation on experimental pneumonia reported in preceding papers^{1,2} a considerable amount of spontaneous pneumonia occurred among the stock monkeys. Since this pneumonia was caused in large part by pneumococci, it seemed desirable to study it for the purpose of comparison with pneumonia experimentally produced. Accordingly, autopsies were performed in all fatal cases and cultures were made from the heart's blood, lungs, and bronchi. In a few instances clinical study of the course of the spontaneous disease was carried out, the same methods being applied that were used in the study of experimental pneumonia. The spontaneous pneumonia also presented certain epidemiological features of considerable interest.

Epidemiology.

The monkeys among which spontaneous pneumonia occurred came in three lots at monthly intervals directly from the Philippine Islands to the Army Medical School in Washington. Experience has shown that the transportation of monkeys is usually attended by a considerable mortality *en route*. Every possible care, therefore, was taken to see that the monkeys were properly handled during their journey, and with the exception of one lot the majority arrived in good condition. Upon their arrival those that appeared healthy were placed in a large, well lighted, and well ventilated cage. Those that appeared sick were placed in small individual cages. Since a

¹ Blake, F. G., and Cecil, R. L., *J. Exp. Med.*, 1920, xxxi, 403.

² Blake, F. G., and Cecil, R. L., *J. Exp. Med.*, 1920, xxxi, 445.

considerable amount of spontaneous pneumonia had occurred among the monkeys of the second lot at the time the third lot arrived, the monkeys of the third lot were placed in pairs in small cages in another room and were not allowed to come into contact with monkeys of the second lot until the outbreak of spontaneous pneumonia had subsided.

The number of monkeys in each lot, the date of their arrival, and the number of recognized cases of pneumonia occurring in each lot are shown in Table I.

The high incidence of pneumonia in Lots 1 and 2, as compared with its total absence among monkeys of Lot 3, is striking. It is of further interest that five of the cases of pneumonia occurring among monkeys of Lot 1 developed during or subsequent to the outbreak of pneumonia among monkeys of Lot 2, monkeys of these two lots being

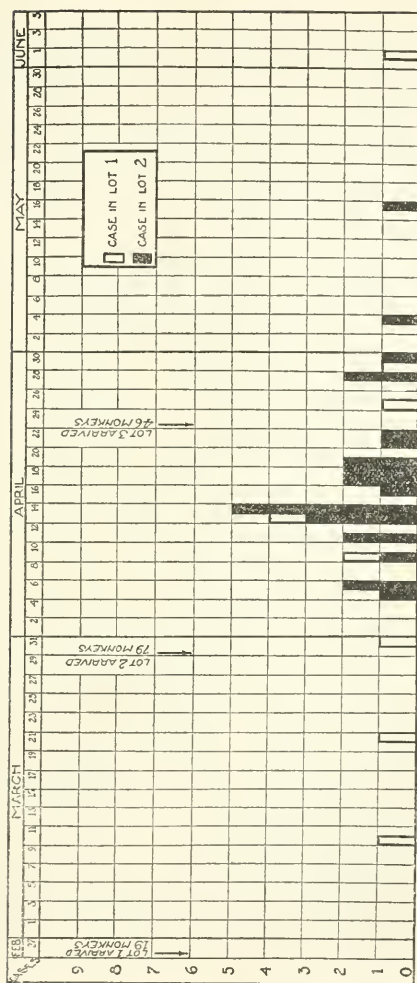
TABLE I.

Spontaneous Pneumonia among Three Lots of Monkeys.

Lot No.	Date of arrival.	No. of monkeys.	No. of cases of pneumonia.	Remarks.
1	1919 Feb. 26	19	8	Five of these cases occurred during or after the outbreak of pneumonia among monkeys of Lot 2.
2	Mar. 30	79	28	Overcrowded.
3	Apr. 23	46	0	Kept separate from preceding lots.

in intimate contact with one another. The absence of pneumonia among the monkeys of Lot 3 undoubtedly may be attributed to the fact that they were not allowed to come into contact with the other monkeys and were kept in pairs in separate cages. That a considerable number of unrecorded cases of mild pneumonia with recovery occurred among the monkeys of Lot 2 was subsequently shown by the finding of fibrous pleural adhesions in many monkeys of this lot used later for experimental purposes.

The apparently epidemic character of the spontaneous pneumonia and its relation to the time of arrival of the different lots of monkeys is shown in Text-fig. 1. It will be seen at once that the larger part of the pneumonia occurred shortly after the arrival of the second lot, the curve of incidence being characteristic of an epidemic outbreak



TEXT-FIG. 1. Spontaneous pneumonia among monkeys from February 26 to June 5, 1919.

of disease. The original source of the outbreak of pneumonia could not be determined. That its spread was due chiefly to contact infection is clearly brought out by bacteriological evidence presented below. It is noteworthy that this was the largest lot of monkeys received and that they were under distinctly crowded conditions during the first 3 weeks after their arrival. There seems little doubt that overcrowding greatly facilitated the spread of infection.

Bacteriology.

Bacteriological examination was made in 36 cases—in 2 cases by blood cultures during life, in 2 cases by blood cultures during life and by cultures at autopsy, and in 32 cases by cultures at autopsy alone (Table II). In 32 cases the results were entirely satisfactory, in 2 no growth was obtained, and in 2 the cultures were grossly contaminated by postmortem invaders and had to be discarded. Of the 32 cases in which bacteriological examinations were satisfactory, *Pneumococcus* Type IV was found in cultures from the lungs or heart's blood, or both, in 28; *Streptococcus hemolyticus* was found in 2; and a non-hemolyzing streptococcus of the *viridans* group in pure culture in 2 and with *Streptococcus hemolyticus* in 1. Of course, it remains problematical whether the cases of pneumonia in which streptococci were found were primarily caused by these organisms, or whether the streptococci were secondary invaders which had replaced the pneumococcus at the time of death. Histological study, however, of the 4 cases in which streptococci were found indicated that the lung lesions were at least in part caused by these organisms. Of the 24 cases of fatal pneumococcus pneumonia in which cultures of the heart's blood were made at autopsy, all but 2 showed the pneumococcus present. In the 2 cases associated with *Streptococcus hemolyticus*, this organism was present in the heart's blood in both, while in the 2 cases associated with *Streptococcus viridans*, cultures of the heart's blood remained sterile in both.

Because of the apparently epidemic character of a considerable part of the spontaneous pneumonia that occurred among the monkeys, it seemed of importance to determine whether or not the strains of *Pneumococcus* Type IV were immunologically homologous. Accord-

TABLE II.

Bacteriology and Pathology of Spontaneous Pneumonia in Monkeys.

Monkey No.	Date.	Bacteriology.	Anatomical diagnosis.
	1919		
11	Mar. 10	H. B., Pn. IV; L. L., no growth; pericard., Pn. IV.	Lobar pneumonia; L. L., resolving; fibrous pleuritis, left; acute fibrinopurulent pericarditis; acute suppurative meningitis; hypertrophy and dilatation of heart.
12	" 21	H. B., Pn. IV; R. L., Pn. IV.	Lobar pneumonia; R. U., R. M., R. L., engorgement and red hepatization.
28	" 31	H. B., Pn. IV; L. M., Pn. IV; R. L., Pn. IV.	Lobar pneumonia; R. M., R. L., L. M., L. L., red and gray hepatization; acute fibrinous pleuritis, bilateral.
36	Apr. 5	H. B., <i>S. hemolyticus</i> ; R. L., <i>S. hemolyticus</i> and <i>S. viridans</i> .	Confluent lobular pneumonia, R. L.; acute fibrinous pleuritis, interlobar, right.
37	" 6	H. B., Pn. IV.	Lobar pneumonia; L. U., L. M., R. M., R. L., gray hepatization; acute fibrinous pleuritis, bilateral.
33	" 6	Contaminated.	Lobar pneumonia; R. M., red hepatization.
15	" 9	L. L., Pn. IV.	Lobar pneumonia; L. L., gray hepatization; acute fibrinous pleuritis, left.
35	" 9	Br., Pn. IV; R. L., Pn. IV.	Lobar pneumonia; R. U., R. M., R. L., gray hepatization; acute fibrinous pleuritis, right.
43	" 11	H. B., Pn. IV; lung, contaminated.	Lobar pneumonia; L. M., L. L., gray hepatization; R. M., red hepatization; acute fibrinous pleuritis, bilateral.
44	" 11	H. B., Pn. IV; L. L., Pn. IV.	Lobar pneumonia; L. L., red hepatization; acute fibrinous pleuritis, left.
52	" 13	H. B., <i>S. hemolyticus</i> ; L. L., no growth.	Lobar pneumonia; L. L., L. M., L. U., gray hepatization and beginning resolution; acute fibrinous pleuritis, left.
53	" 13	H. B., no growth; L. M., Pn. IV; C., Pn. IV; Br., Pn. IV.	Lobar pneumonia; R. M., gray hepatization; C., L. M., red hepatization; R. U., engorgement; acute fibrinous pleuritis, right.
54	" 13	H. B., Pn. IV; R. L., Pn. IV.	Lobar pneumonia; R. L., gray hepatization; acute fibrinous pleuritis, right.

H. B. indicates heart's blood; B. C., blood culture; R. L., C., L. L., etc., lobes of the lung; Pl., pleura; Br., bronchus.

TABLE II—Continued.

Monkey No.	Date.	Bacteriology.	Anatomical diagnosis.
	1919		
18	Apr. 13	H. B., no growth; R. L., <i>S. viridans</i> ; L. M., <i>S. viridans</i> .	Interstitial and lobular pneumonia, R. M., R. L., L. L.; acute fibrinous pleuritis, bilateral.
55	" 14	H. B., Pn. IV; L. L., Pn. IV.	Lobar pneumonia; R. M., R. L., L. M., L. L., red and gray hepatization; acute fibrinous pleuritis, bilateral.
56	" 14	H. B., Pn. IV; L. L., Pn. IV.	Lobar pneumonia; L. L., gray hepatization; acute fibrinous pleuritis, left.
57	" 14	H. B., no growth; L. L., no growth.	Lobar pneumonia; L. L., gray hepatization; acute fibrinous pleuritis, left.
58	" 14	Contaminated.	Lobar pneumonia; R. M., R. L., gray hepatization; acute fibrinous pleuritis, right.
59	" 14	H. B., Pn. IV; L. U., no growth.	Lobar pneumonia; L. U., L. M., gray hepatization; R. M., resolution; acute fibrinous pleuritis, bilateral.
34	" 16	H. B., Pn. IV; L. L., no growth; R. L., contaminated.	Lobar pneumonia; L. U., L. M., engorgement; L. L., gray hepatization; R. L., resolution; acute fibrinous pleuritis, bilateral.
60	" 17	H. B., no growth; L. L., <i>S. viridans</i> .	Interstitial and lobar pneumonia; R. U., R. M., R. L., gray hepatization; L. L., interstitial and lobular pneumonia; acute fibrinous pleuritis, bilateral.
61	" 17	H. B., Pn. IV; L. L., Pn. IV.	Lobar pneumonia; L. U., L. M., L. L., red and gray hepatization; acute fibrinous pleuritis, left.
62	" 18	H. B., Pn. IV; L. L., no growth.	Lobar pneumonia; R. M., C., red hepatization; L. L., resolution; acute fibrinous pleuritis, left.
50	" 18	H. B., Pn. IV; L. M., Pn. IV; L. L., Pn. IV.	Lobar pneumonia; L. M., L. L., gray hepatization; L. U., C., red hepatization; acute fibrinous pleuritis, left.
68	" 19	H. B., Pn. IV; R. L., contaminated.	Lobar pneumonia; R. M., R. L., L. M., red hepatization; acute fibrinous pleuritis, right.
69	" 19	H. B., Pn. IV; L. U., Pn. IV.	Lobar pneumonia; L. U., L. M., gray hepatization; acute fibrinous pleuritis, left.
70	" 21	H. B., Pn. IV; R. M., Pn. IV.	Lobar pneumonia; L. U., L. M., gray hepatization; R. M., R. L., red hepatization; acute fibrinous pleuritis, bilateral.

TABLE II—*Concluded.*

Monkey No.	Date.	Bacteriology.	Anatomical diagnosis.
	1919		
74	Apr. 22	H. B., Pn. IV; L. L., no growth.	Lobar pneumonia; L. L., resolution; R. M., R. L., red hepatization; acute fibrinous pleuritis, bilateral.
13	" 25	H. B., Pn. IV; R. L., no growth; pericard., Pn. IV.	Lobar pneumonia; R. M., R. L., organization; L. M., L. L., resolution and organization; acute fibrinous pleuritis, bilateral; acute serofibrinous pericarditis; hypertrophy and dilatation of heart.
71	" 28	H. B., no growth; L. U., Pn. IV.	Lobar pneumonia; L. U., gray hepatization; acute fibrinous pleuritis, left.
73	" 28	H. B., Pn. IV; L. L., no growth.	Lobar pneumonia; L. M., L. L., C., R. M., R. L., gray hepatization; acute fibrinous pleuritis, bilateral.
21	" 29	L. M., no growth; pericard., no growth.	Lobar pneumonia; L. U., L. M., resolution; acute fibrinous pleuritis, left; acute fibrinous pericarditis.
47	" 30	B. C., Pn. IV.	Lobar pneumonia; L. M., L. L., gray hepatization and resolution; R. M., resolution; acute fibrinous pleuritis, left.
84	May 4	H. B., Pn. IV; R. L., contaminated	Lobar pneumonia; L. L., resolution; R. L., C., gray hepatization; acute fibrinous pleuritis, bilateral.
97	" 16	H. B., Pn. IV; L. L., Pn. IV; Pl., Pn. IV; pericard., Pn. IV; Br., Pn. IV.	Lobar pneumonia; R. U., R. M., R. L., C., L. M., L. L., red and gray hepatization; acute fibrinopurulent pleuritis, bilateral; acute fibrinopurulent pericarditis; acute fibrinous peritonitis, localized.
19	June 1	B. C., Pn. IV.	(Clinical diagnosis, lobar pneumonia. Recovered; no autopsy.)

ingly, three rabbits were immunized against the strains isolated from Monkeys 19, 53, and 97, respectively, and the immune sera so prepared were used for cross-agglutination tests. Of the twenty-eight strains isolated, twenty-one had been preserved and were available for agglutination. The results are shown in Table III.

It will be seen from Table III that nine strains (from Monkeys 13, 19, 34, 35, 47, 73, 74, 84, and 97) fell into one immunologically homologous group, that five strains (from Monkeys 50, 53, 61, 69, and 70) fell

into another homologous group, and that there remained but seven strains which fell into neither of these groups. Though some of the strains remain unclassified, the evidence obtained is amply sufficient to demonstrate that the outbreak of spontaneous pneumonia was associated with the spread of infection from monkey to monkey. This becomes all the more apparent when the immunological groups are cor-

TABLE III.

Cross-Agglutination Reactions of Strains of Pneumococcus Type IV Isolated from Monkeys with Spontaneous Lobar Pneumonia.

Strain No.	Immune sera.			Normal serum.
	No. 19.	No. 53.	No. 97.	
12	—	—	—	—
13	++	—	++	—
15	—	—	—	—
19	++	—	++	—
28	—	—	—	—
34	++	—	++	—
35	++	—	++	—
44	—	—	—	—
47	++	—	++	—
50	—	++	—	—
53	—	++	—	—
55	—	—	—	—
56	—	—	—	—
61	—	++	—	—
69	—	++	—	—
70	—	++	—	—
71	—	—	—	—
73	++	—	++	—
74	++	—	++	—
84	++	—	++	—
97	++	—	++	—

related with the dates of death from pneumonia in the individual cases. The strains isolated from Monkeys 12 and 28 did not fall into either of the established groups. Both these monkeys were from Lot 1 and developed pneumonia before the arrival of Lot 2, dying on March 21 and 31, respectively. Monkeys 53, 61, 50, 69, and 70, whose pneumonia was due to the same strain of *Pneumococcus* Type IV, died on April 13, 17, 18, 19, and 21, respectively, within a period of 9 days.

Of the nine cases of pneumonia due to the other single strain of *Pneumococcus* Type IV, Monkeys 35, 34, 74, 13, 73, 84, and 97 died on April 9, 16, 22, 25, 28, May 4, and 16, respectively. Monkey 47 developed pneumonia on April 30 and recovered by crisis on May 9. These eight cases were sufficiently near to each other to have contracted pneumonia by contact infection from a preceding case. The ninth case caused by a pneumococcus of this type, Monkey 19, developed pneumonia on June 1, and had, therefore, no direct relation with the epidemic. Monkey 19 was at the time, however, in a cage adjoining that of Monkey 111. At this time Monkey 111 was in the active stage of an experimental pneumonia produced by inoculation with the strain of *Pneumococcus* Type IV which had been isolated at necropsy from Monkey 97 of this group. It is presumable that Monkey 19 acquired its spontaneous infection from this source.

Clinical Features.

Clinical observations upon spontaneous lobar pneumonia were made in two instances. In a third case the course of pneumococcus pericarditis following spontaneous pneumonia was studied. The protocols with accompanying clinical charts follow.

Protocol 1.—Monkey 47. *Macacus syrichtus*, male; weight 2,710 gm. Apr. 29, 1919. Well and active. Apr. 30, a.m. Well and active. p.m. Appears quiet. May 1. Appears sick. Respirations moderately accelerated. Temperature elevated. No abnormal signs in chest. May 2. Very sick; sitting huddled up in corner of cage, breathing rapidly with expiratory grunt; blood culture shows *Pneumococcus* Type IV. May 3. Condition the same; moderate dullness and suppressed breathing over left lower lobe; distant bronchial breathing heard in left axilla. May 4. Appears better; respirations rapid and labored. May 5 to 8. Condition the same; coughs occasionally; breathing rapidly. May 9. Appears well and active; temperature fallen to normal by crisis. May 10. Well and active. Killed.

The temperature, leucocyte curve, and results of blood cultures are shown in Text-fig. 2.

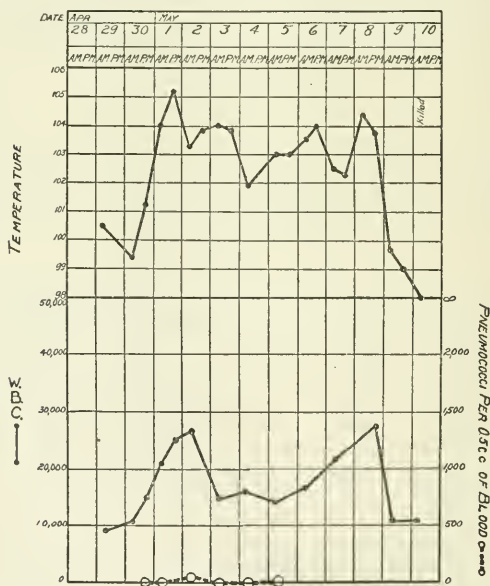
Autopsy. Anatomical Diagnosis.—Lobar pneumonia; left middle and lower lobes, gray hepatization and beginning resolution; right middle lobe resolving; acute fibrinous pleuritis, left.

Cultures.—Heart's blood, no growth; left lower lobe, no growth.

Protocol 2.—Monkey 19. *Macacus syrichtus*, male; weight 4,000 gm. May 29 to 31, 1919. Appears well and active. June 1. Quiet; temperature elevated; breathing rapidly; leucocytosis. June 2. Appears sick; refuses food; respirations rapid and labored; blood culture shows *Pneumococcus* Type IV. June 3 to 5. Condition the same. June 6. Appears better; still breathing rapidly. June 7. Better; temperature falling. June 8. Appears well and active; temperature normal. Remained well. No autopsy.

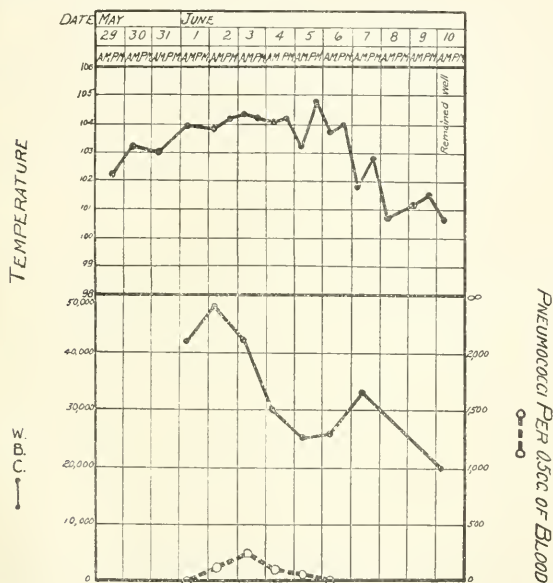
The temperature, leucocyte curve, and results of blood cultures are shown in Text-fig. 3.

Both monkeys presented the typical picture and ran the characteristic course of lobar pneumonia, identical with the disease experi-



TEXT-FIG. 2. Monkey 47. Spontaneous lobar pneumonia, *Pneumococcus* Type IV.

mentally produced by the intratracheal injection of pneumococcus, and like it, corresponding in all respects to lobar pneumonia in man. The leucocyte reaction, characteristic of relatively mild cases that recover, is well shown, as is the temporary invasion of the blood by pneumococci and recovery by crisis.

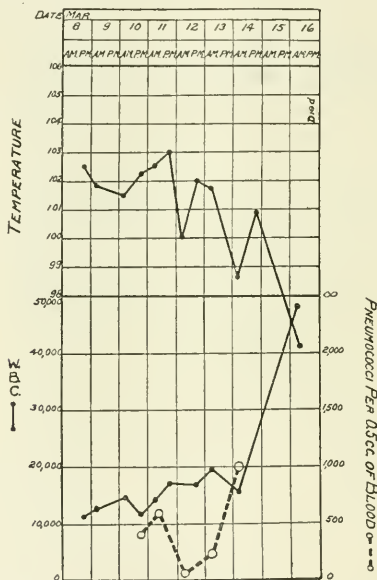


TEXT-FIG. 3. Monkey 19. Spontaneous lobar pneumonia, *Pneumococcus* Type IV.

Protocol 3.—Monkey 11. *Macacus syrichtus*, male; weight 4,325 gm. Mar. 8, 1919. Appears moderately sick; breathing slightly accelerated; lungs clear. X-ray of chest shows cardiac shadow moderately enlarged and distinctly globular; lungs show no evidence of consolidation (Fig. 1). Mar. 10. Condition the same. Blood culture shows *Pneumococcus* Type IV. Mar. 11 to 13. Condition the same. Lungs clear. Mar. 14. Right leg appears paralyzed. Mar. 16. Very sick; lying down; semicomatose. 9.30 p.m. Died.

The temperature, leucocyte curve, and results of blood cultures are shown in Text-fig. 4.

Autopsy. Anatomical Diagnosis.—Resolving lobar pneumonia, left lower lobe; acute fibrinopurulent pericarditis; acute suppurative meningitis; hypertrophy and dilatation of the heart; fibrous pleuritis, left.



TEXT-FIG. 4. Monkey 11. Fibrinopurulent pericarditis with terminal meningitis following spontaneous lobar pneumonia, *Pneumococcus* Type IV.

Pericardium.—Appears considerably thickened, and on opening the sac a considerable quantity of turbid fluid is found. The parietal and visceral layers of the pericardium are everywhere covered with a shaggy coat of fibrin which is easily peeled off from the underlying membrane. *Heart.*—Moderate hypertrophy and dilatation. *Left pleural cavity.*—Contains many fibrous adhesions between the parietal and visceral pleuræ. *Lungs.*—Crepitant and pale pink, except the left lower lobe which is a translucent gray and doughy. *Brain.*—Appears normal.

Cultures.—Heart's blood, *Pneumococcus* Type IV; pericardial exudate, *Pneumococcus* Type IV; left lower lobe, no growth.

Microscopic Examination.—*Heart.*—Myocardium shows cloudy swelling; on the epicardium there is a dense layer of fibrin everywhere infiltrated with polymorphonuclear leucocytes and lymphoid cells. *Left lower lobe.*—Shows the last stages of a resolving pneumonia. *Medulla.*—The meninges are densely infiltrated with polymorphonuclear leucocytes, which form a thick layer over the surface.

There seems little question that the pneumococcus pericarditis in Monkey 11 developed following spontaneous lobar pneumonia. It is présumable that the terminal meningitis was also caused by *Pneumococcus* Type IV, although bacteriological evidence is lacking. Both conditions were similar to pneumococcus pericarditis and meningitis as seen in man complicating lobar pneumonia.

Pathology.

Thirty-two cases presented at autopsy the characteristic pathology of pneumococcus lobar pneumonia (Table II), resembling in all respects the experimental disease produced by the intratracheal injection of pneumococci as described in a preceding paper.² *Pneumococcus* pericarditis occurred in four cases, empyema in one, and meningitis in one. Acute fibrinous pleuritis was present in all but two cases. In the majority of cases the pneumonia was extensive, more than one lobe being involved in twenty-four cases; seventeen cases showed bilateral pneumonia. A characteristic feature of these cases was the presence of an advanced stage of gray hepatization or beginning resolution in some lobes, while in others the picture of engorgement and red hepatization was found, indicating that a spread of the process had occurred during the course of the disease. In eight cases only one lobe was involved, in one of which death was due to a complicating pericarditis. The right and left sides were about equally involved, the lower and middle lobes nearly four times as frequently as the upper lobes. As in experimental pneumonia in monkeys partial organization occurred somewhat more frequently than it is believed to occur in lobar pneumonia in man. Macroscopically the cut surfaces of the lungs presented the characteristic appearances of the stages of engorgement, red and gray hepatization, and beginning resolution. The lobar character of the consolidation

is well shown in photographs of the lungs from Monkeys 43 and 57 (Figs. 2 and 3) and of a stained histological section of the right lower lobe from Monkey 84 (Fig. 4). Location of the consolidation in the proximal portion of the lobe when complete lobar consolidation did not develop is shown in Fig. 5. Cases associated with streptococci are too few in number to warrant description. The character of the lesion in Monkey 36 is shown in Fig. 6.

In a similar manner study of histological sections (Figs. 7 to 16) has shown the spontaneous disease to be identical with that experimentally produced, leaving no doubt that the pathogenesis of the two is the same. A few illustrative protocols are given.

Protocol 4.—Monkey 35. *Macacus syrichtus*, female. Autopsy performed Apr. 9, 1919, 8 hours post mortem.

Anatomical Diagnosis.—Lobar pneumonia; right upper, middle, and lower lobes, stage of gray hepatization; acute fibrinous pleuritis, right; cloudy swelling of viscera.

Pericardial sac.—Contains no fluid and appears normal. *Heart.*—Of normal size; myocardium firm; valves normal. *Pleural cavities.*—Contain no fluid. There is a fresh deposit of fibrin on the visceral and parietal layers of the right pleura. The left pleura is smooth and glistening. *Lungs.*—The right upper, middle, and lower lobes are completely consolidated, except for a small portion along the anterior margins; surfaces dark red and roughened with fibrin. The lobes are plastered together with a fibrinous exudate. Cut surface of the right lung is reddish gray and finely granular. Left lung is pale pink, somewhat collapsed, and crepitant throughout. The trachea and bronchi are filled with rusty mucoid material. *Other organs.*—Abdominal organs normally disposed; liver and kidneys show cloudy swelling.

Cultures.—Right lower lobe, *Pneumococcus* Type IV; right bronchus, *Pneumococcus* Type IV.

Microscopic Examination.—*Right lower lobe.*—Except at the periphery the alveoli are filled with a dense exudate of polymorphonuclear leucocytes, the structure of the alveolar walls being well preserved (Fig. 7). The perivascular tissue about the larger vessels is edematous and moderately infiltrated with polymorphonuclear leucocytes, large mononuclear cells, and lymphocytes, the lymphatics being distended with similar cells (Fig. 8). The bronchi and bronchioles are filled with exudate. At the periphery there is marked congestion of the smaller vessels and alveolar capillaries and perivascular edema. The alveoli contain a serous exudate with few cells (Fig. 9). There is a dense exudate of polymorphonuclear leucocytes and fibrin on the pleural surface. There are many pneumococci in all parts of the section, both in the alveoli and in the alveolar walls at the periphery.

Protocol 5.—Monkey 53. *Macacus syrichtus*, male. Autopsy performed Apr. 13, 1919.

Anatomical Diagnosis.—Lobar pneumonia; right middle lobe, gray hepatization; cardiac lobe and left middle lobe, red hepatization; right upper lobe, engorgement and edema; acute fibrinous pleuritis, right.

Pericardial sac.—Contains no fluid and appears normal. *Heart.*—Markedly enlarged, both ventricles being dilated; myocardium and valves appear normal. *Pleural cavities.*—Contain no fluid. There is a deposit of fibrin on the pleural surfaces in the lower part of the right pleural cavity. The left pleura is smooth and glistening. *Lungs.*—The right middle lobe is firmly consolidated throughout, the cut surface being yellowish gray and finely granular. The right upper lobe shows a small patch of beginning consolidation near the hilum, deep red in color, the cut surface oozing abundant frothy fluid. The cardiac lobe is moderately firm throughout, deep red in color, the cut surface moist. The left middle lobe shows a similar picture. The right lower, left upper, and left lower lobes are pale pink and crepitant throughout. *Abdominal organs.*—Show nothing of interest.

Cultures.—Heart's blood, no growth; left middle lobe, *Pneumococcus* Type IV; cardiac lobe, *Pneumococcus* Type IV; bronchus, *Pneumococcus* Type IV.

Microscopic Examination.—*Right middle lobe.*—The alveoli are everywhere filled with a dense exudate of polymorphonuclear leucocytes; in some places these show marked disintegration and there is beginning desquamation of alveolar epithelium. There is moderate perivascular edema and infiltration with polymorphonuclear leucocytes about some of the smaller vessels. Many pneumococci are present in the pneumonic exudate. *Right upper lobe.*—Central portion shows marked engorgement of vessels and alveolar capillaries, serous exudate with fibrin network in the alveoli, and some exudation of red blood corpuscles; tissue appears normal at the periphery. Pneumococci are numerous in the walls of the alveoli and in the alveolar exudate. They are also frequently seen in the apparently uninvolved peripheral portions of the lobe where they are confined within the walls of the alveoli.

Protocol 6.—Monkey 97. *Macacus syrichtus*, female. Autopsy performed May 16, 1919, immediately after death.

Anatomical Diagnosis.—Lobar pneumonia; right lower, middle, cardiac, and upper lobes, left lower and middle lobes, red and gray hepatization; acute fibrinopurulent pericarditis; acute fibrinopurulent pleuritis, bilateral; acute fibrinous peritonitis, localized; cloudy swelling of viscera.

Pericardial sac.—Contains about 10 cc. of yellowish, turbid fluid, in which are flakes of fibrin. Visceral and parietal layers of the pericardium are covered with a shaggy fibrinous exudate. *Heart.*—Of normal size; myocardium and valves appear normal. *Pleural cavities.*—Both contain about 25 cc. of turbid, yellowish fluid. There is abundant fibrinous exudate over both lower and middle lobes. *Lungs.*—The right middle and lower lobes are firmly consolidated throughout, the cut surfaces being reddish gray in the center, deep red and very moist at

the periphery. The upper half of the cardiac lobe and a small portion of the right upper lobe near the hilum present a similar appearance. The left middle lobe and the upper and posterior half of the left lower lobe are firmly consolidated, the cut surfaces reddish gray and finely granular. The remainder of the lungs is pinkish and air-containing, but the cut surfaces yield a large amount of frothy fluid. Trachea and bronchi are filled with bloody mucus. *Other organs.*—There are a few fresh fibrinous adhesions between the diaphragm and the upper surface of the liver. Otherwise abdominal organs appear normal, except for cloudy swelling of liver and kidneys.

Cultures.—Heart's blood, *Pneumococcus* Type IV; pericardial fluid, *Pneumococcus* Type IV; right pleural fluid, *Pneumococcus* Type IV; left lower lobe, *Pneumococcus* Type IV; bronchus, *Pneumococcus* Type IV.

Microscopic Examination.—*Left lower lobe.*—Section shows typical picture of gray hepatization, the alveoli being filled with a dense exudate of polymorphonuclear leucocytes. The structure of the alveolar walls is well preserved. The peribronchial and perivascular tissue is edematous and infiltrated with leucocytes and mononuclear cells (Fig. 11). At the periphery there is a zone of congestion and edema, the alveoli containing only a serous exudate. In places the alveoli are filled with red blood corpuscles. The right lower lobe presents a similar picture. *Pneumococci* are plentiful in the exudate and in the edematous alveoli. They are also found in considerable numbers in the alveolar walls in uninvolved portions of the lung near the periphery. In such areas they are not found in the alveolar lumen. *Left pleura.*—Thick exudate of fibrin and leucocytes (Fig. 12).

Protocol 7.—Monkey 13. *Macacus syrichtus*, male. Autopsy performed Apr. 25, 1919, 12 hours post mortem.

Anatomical Diagnosis.—Lobar pneumonia; right middle and lower lobes, organization; left middle and lower lobes, resolution and organization; acute serofibrinous pericarditis; acute fibrinous pleuritis, bilateral; hypertrophy and dilatation of heart.

Pericardial sac.—Contains a slightly increased amount of cloudy fluid. *Heart.*—Greatly enlarged, the right auricle and ventricle being markedly dilated; myocardium and valves show no abnormalities. *Pleural cavities.*—Contain no fluid. There are firm fibrinous adhesions in both pleural cavities which show beginning organization. *Lungs.*—Entire left lung is flabby and has a rubbery consistency. Upper and middle lobes are firmly bound together; cut surface is pinkish, moist, and for the most part air-containing. The right middle and lower lobes are firmly consolidated, presenting on section a uniform, grayish, translucent surface. *Abdominal organs.*—Show cloudy swelling.

Cultures.—Heart's blood, *Pneumococcus* Type IV; pericardial fluid, *Pneumococcus* Type IV; right lower lobe, no growth.

Microscopic Examination.—*Right lower lobe.*—Section shows advanced organization. There is almost complete absence of exudate in the alveoli, and the latter have been greatly compressed and distorted. Some alveoli show marked

dilatation. In many places the alveolar walls are greatly thickened and the alveoli are filled with plugs of new formed connective tissue which is vascular and is infiltrated with lymphocytes (Fig. 14). There is marked perivascular organization with infiltration of lymphocytes and plasma cells. Similar changes are noted around the bronchi, though less marked. No bacteria are seen. *Left lower lobe.*—Presents a similar picture.

The foregoing protocols clearly show that the pathology of spontaneous lobar pneumonia in monkeys is identical with that of lobar pneumonia experimentally produced. Since monkeys which died of spontaneous pneumonia had reached a well advanced stage of the disease, the purely interstitial character of the stage of invasion, as described in connection with the pathogenesis of experimental pneumonia, was not encountered. The frequent occurrence, however, even in the later stages of varying degrees of perivascular edema and infiltration, the presence of pneumococci in the alveolar walls of uninvolved peripheral portions of the lung, and the more advanced character of the pneumonic process in the areas about the larger vessels and bronchi can leave little doubt that the mode of involvement of the lung was the same as that which occurs when pneumococci are experimentally injected into the trachea.

SUMMARY.

Spontaneous pneumonia occurred to a considerable extent among stock monkeys at the Army Medical School. This pneumonia occurred chiefly in the form of an epidemic outbreak shortly after the arrival of a large shipment of monkeys, and was shown to be due in large part to transmission of infection from monkey to monkey, either directly or indirectly. That spread of the epidemic was facilitated by overcrowding was indicated by the fact that in a subsequent shipment of monkeys, which were kept in pairs in separate cages and were not allowed to come into contact with the monkeys among which the epidemic occurred, no cases of spontaneous pneumonia developed.

The close analogy between the epidemic of lobar pneumonia that occurred among the monkeys and similar epidemics of lobar pneumonia that occurred during the war among certain groups of newly drafted troops shortly after their arrival at camp is very striking,

and would seem to indicate that pneumococcus pneumonia may become an epidemic disease among groups of susceptible individuals when they are assembled under conditions that facilitate the ready transfer of infection from individual to individual.

Bacteriological examination showed the spontaneous pneumonia to be due in the great majority of cases to infection with *Pneumococcus* Type IV. Immunological classification of the strains of pneumococci by cross-agglutination tests showed that the majority fell into two biological groups. Two cases were apparently caused by *Streptococcus hæmolyticus*, two by *Streptococcus viridans*.

The clinical course of spontaneous pneumococcus pneumonia in monkeys was characterized by sudden onset, high sustained temperature, leucocytosis, rapid respiration with expiratory grunt, cough, physical signs of consolidation, invasion of the blood by pneumococci, and termination in death or recovery by crisis about the 7th to 9th day. In a few instances the disease was complicated by acute fibrinopurulent pericarditis, by empyema, and by purulent meningitis. It was, therefore, clinically identical with lobar pneumonia experimentally produced in monkeys and with lobar pneumonia in man.

Study of the pathology of spontaneous pneumococcus pneumonia in monkeys showed that it presented the characteristic picture of lobar pneumonia, both macroscopically and microscopically, and was in all respects comparable with the pathology of lobar pneumonia experimentally produced in monkeys and of lobar pneumonia in man.

CONCLUSIONS.

1. Monkeys in captivity are subject to spontaneous lobar pneumonia.
2. Spontaneous lobar pneumonia in monkeys is caused by the pneumococcus.
3. Lobar pneumonia in monkeys may occur as an epidemic disease and be due to spread of infection from animal to animal when conditions which favor contact infection are present.
4. Spontaneous lobar pneumonia in monkeys is identical in its clinical features, complications, and pathology with lobar pneumonia experimentally produced in monkeys by the intratracheal injection of pneumococcus and with lobar pneumonia in man.

EXPLANATION OF PLATES.

PLATE 53.

FIG. 1. Monkey 11. Fibrinopurulent pericarditis following spontaneous lobar pneumonia, *Pneumococcus* Type IV. X-ray of chest.

PLATE 54.

FIG. 2. Monkey 43. Spontaneous lobar pneumonia, *Pneumococcus* Type IV. Cut surface of lungs showing gray hepatization of left lower and middle lobes; red hepatization of right middle lobe.

PLATE 55.

FIG. 3. Monkey 57. Spontaneous lobar pneumonia, *Pneumococcus* Type IV. Cut surface of lungs showing gray hepatization and beginning resolution of left lower lobe.

PLATE 56.

FIG. 4. Monkey 84. Spontaneous lobar pneumonia, *Pneumococcus* Type IV. Section completely through right lower lobe showing lobar character of consolidation; stage of gray hepatization.

FIG. 5. Monkey 59. Spontaneous lobar pneumonia, *Pneumococcus* Type IV. Section completely through right middle lobe showing hepatization of the part of the lobe proximal to the hilum, perivascular edema and infiltration, and peribronchial and interstitial involvement with absence of exudate in the alveoli and bronchioles in the distal portion of the lobe.

PLATE 57.

FIG. 6. Monkey 36. Spontaneous pneumonia, *Streptococcus hemolyticus*. Section completely through the right lower lobe.

PLATE 58.

FIG. 7. Monkey 35. Spontaneous lobar pneumonia, *Pneumococcus* Type IV. Section from right lower lobe, stage of gray hepatization, showing alveoli filled with leucocytic exudate. $\times 100$.

PLATE 59.

FIG. 8. Monkey 35. Spontaneous lobar pneumonia, *Pneumococcus* Type IV. Section from right lower lobe, stage of gray hepatization, showing perivascular edema and moderate infiltration, perivascular lymphatics filled with leucocytes, and exudate in the lumen of a small bronchus. $\times 100$.

PLATE 60.

FIG. 9. Monkey 35. Spontaneous lobar pneumonia, *Pneumococcus* Type IV. Section from margin of right lower lobe showing vascular and capillary engorgement and serous exudate in alveoli. $\times 100$.

FIG. 10. Monkey 28. Spontaneous lobar pneumonia, *Pneumococcus* Type IV. Section from right lower lobe, stage of red hepatization, showing alveolar exudate of coagulated serum, fibrin, red blood cells, and a few polymorphonuclear leucocytes. $\times 100$.

PLATE 61.

FIG. 11. Monkey 97. Spontaneous lobar pneumonia, *Pneumococcus* Type IV. Section from left lower lobe showing edema and infiltration of bronchial wall. $\times 100$.

PLATE 62.

FIG. 12. Monkey 97. Spontaneous lobar pneumonia, *Pneumococcus* Type IV. Section from left lower lobe showing thick exudate of fibrin and leucocytes on the pleura. $\times 64$.

FIG. 13. Monkey 47. Spontaneous lobar pneumonia, *Pneumococcus* Type IV. Monkey killed 24 hours after recovery by crisis. Section from left lower lobe (beginning resolution) showing intense leucocytic infiltration of perivascular tissue. $\times 100$.

PLATE 63.

FIG. 14. Monkey 13. Spontaneous lobar pneumonia, death resulting from a complicating pneumococcus pericarditis. Section from right lower lobe showing alveoli filled with plugs of young connective tissue and beginning organization of perivascular tissue. $\times 100$.

PLATE 64.

FIG. 15. Monkey 62. Spontaneous lobar pneumonia, *Pneumococcus* Type IV. Section from cardiac lobe showing early alveolar lesion with marked leucocytic infiltration of alveolar walls and in places beginning exudation into the alveoli, the alveolar duct and atrium being free from exudate. $\times 83$.

FIG. 16. Monkey 70. Spontaneous lobar pneumonia, *Pneumococcus* Type IV. Section from right lower lobe showing invasion of alveolar walls by pneumococci beyond the margin of the consolidated area. $\times 1,000$.



FIG. 1.

(Blake and Cecil: Experimental pneumonia. III.)



FIG. 2.

(Blake and Cecil: Experimental pneumonia, III.)

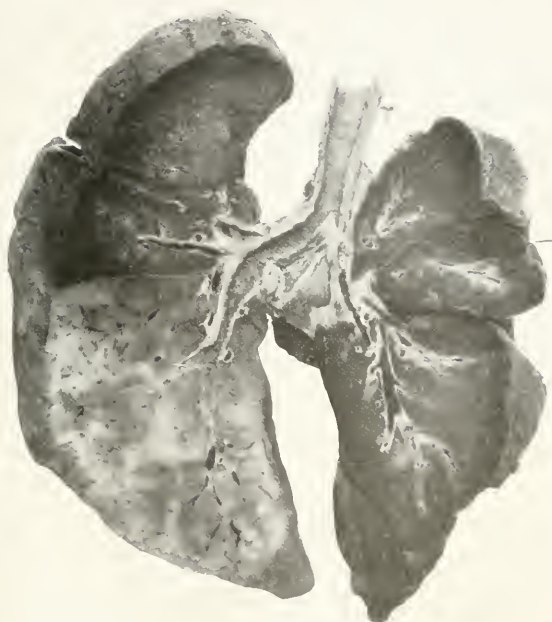


FIG. 3.

(Blake and Cecil: Experimental pneumonia. III.)



FIG. 4.



FIG. 5.



FIG. 6.

(Blake and Cecil: Experimental pneumonia. III.)



FIG. 7.

(Blake and Cecil: Experimental pneumonia. III.)



FIG. 8.

(Blake and Cecil: Experimental pneumonia. III.)

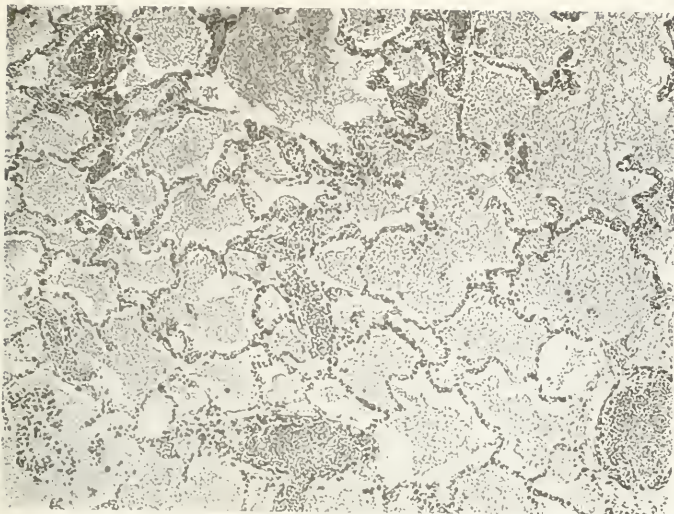


FIG. 9.

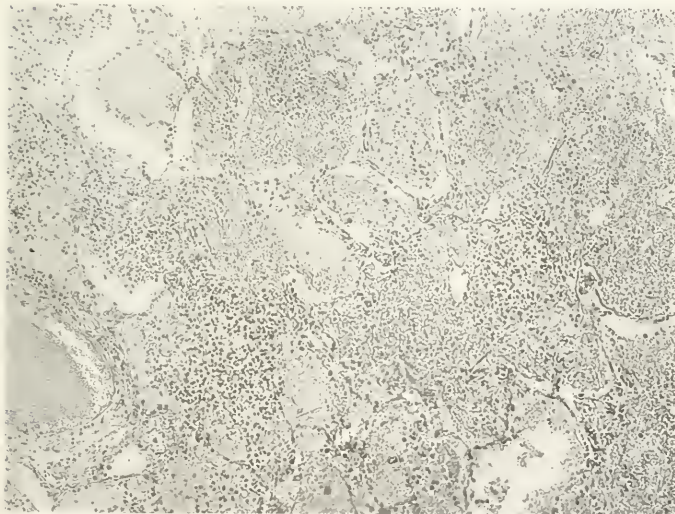


FIG. 10.

(Blake and Cecil: Experimental pneumonia. III.)

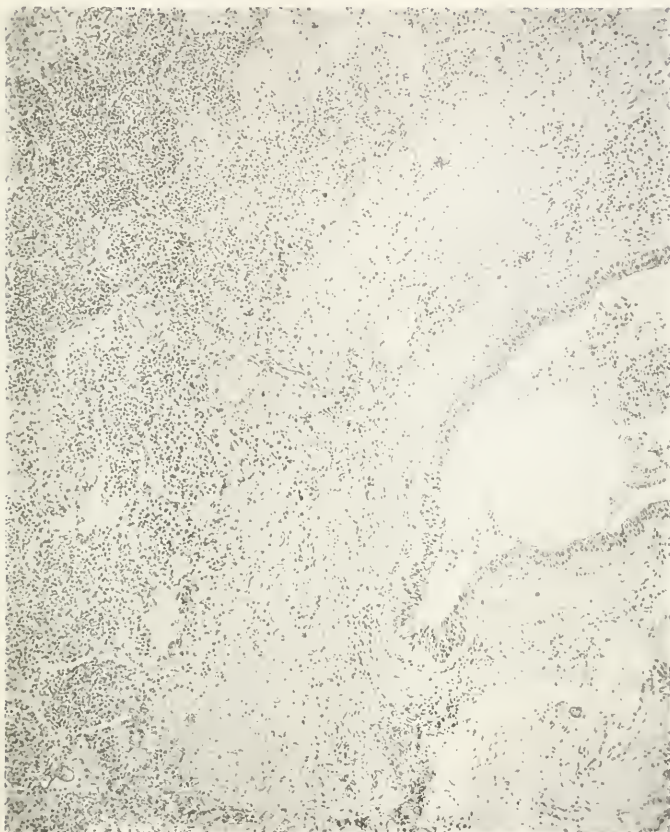


FIG. 11.

(Blake and Cecil: Experimental pneumonia. III.)

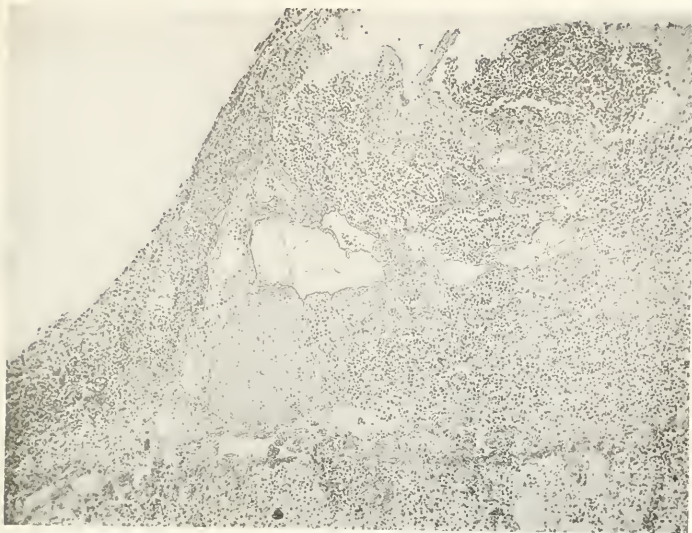


FIG. 12.



FIG. 13.

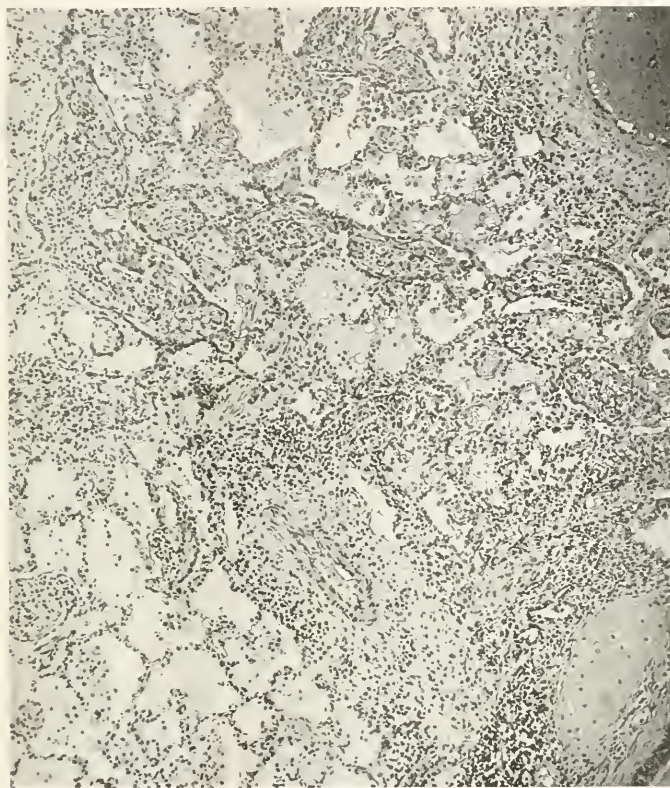


FIG. 14.

(Blake and Cecil: Experimental pneumonia. III.)

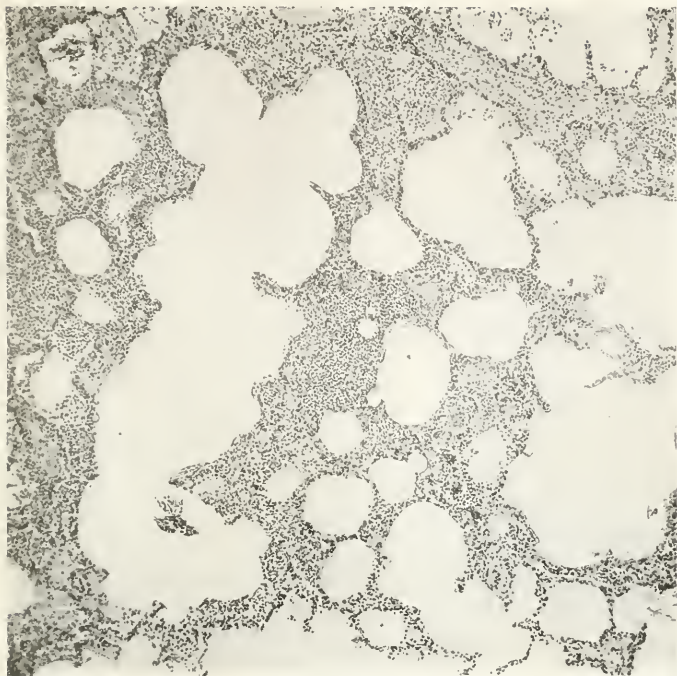


FIG. 15.

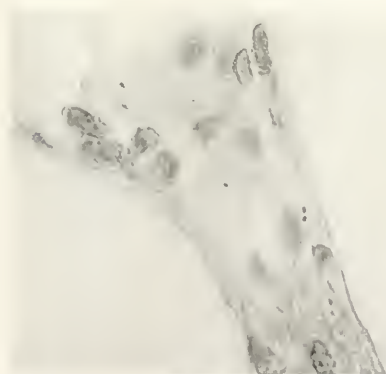


FIG. 16.

(Blake and Cecil: Experimental pneumonia. III.)

STUDIES ON EXPERIMENTAL PNEUMONIA.

IV. RESULTS OF PROPHYLACTIC VACCINATION AGAINST PNEUMOCOCCUS PNEUMONIA IN MONKEYS.

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(Received for publication, January 23, 1920.)

Prophylactic vaccination against pneumonia has been practised with apparent success on the miners in South Africa by Wright,¹ and more recently by Lister.² Within the past 2 years similar investigations have been undertaken in the United States Army Camps by Cecil and Austin³ at Camp Upton, and by Cecil and Vaughan⁴ at Camp Wheeler. The results obtained in these later experiments were so encouraging that the whole question of active immunity against pneumococcus seemed worthy of thorough study.

It has long been recognized that injection into animals of killed cultures of pneumococcus would protect them against lethal doses of living virulent pneumococci injected intravenously. Animals vaccinated in this way usually develop agglutinins and protective substances in their sera. Dochez⁵ has shown that in man protective bodies are usually demonstrable in the serum of a patient immediately following an attack of pneumonia; and Cecil and Austin³ found that the injection of killed pneumococci in man would, in some cases at least, stimulate the production of agglutinins and protective bodies.

¹ Wright, A. E., *Lancet*, 1914, i, 87.

² Lister, F. S., An experimental study of prophylactic inoculation against pneumococcal infection in the rabbit and in man, Publications of the South African Institute for Medical Research, No. 8, Johannesburg, 1916; Prophylactic inoculation of man against pneumococcal infections, and more particularly against lobar pneumonia, Publications of the South African Institute for Medical Research, No. 10, Johannesburg, 1917.

³ Cecil, R. L., and Austin, J. H., *J. Exp. Med.*, 1918, xxviii, 19.

⁴ Cecil, R. L., and Vaughan, H. F., *J. Exp. Med.*, 1919, xxix, 457.

⁵ Dochez, A. R., *J. Exp. Med.*, 1912, xvi, 665.

In spite of the fact that the injection of killed cultures of pneumococcus in rabbits, horses, and other animals will protect these animals against lethal doses of the living organism, this accomplishment is not equivalent to preventing the disease, pneumonia, itself. Indeed, very little is known concerning the whole subject of active immunity against pneumonia and comparatively little experimental work has been done along this line.

Wadsworth⁶ immunized eleven rabbits against pneumococcus by injecting them with pneumococci which had been dissolved in rabbit bile. The eleven immunized rabbits and five controls were then injected intratracheally with 1 cc. of a virulent pneumococcus culture. Of the five control animals, three died in 48 hours without lung lesions; a fourth lived 4 days and a small patch of pneumonia was found at autopsy. The fifth was dying on the 5th day when it was killed. A small area of consolidation was found in this animal also. Of the eleven immunized animals, none died, but a few were seriously ill from 24 to 36 hours. All the immunized animals, when killed, showed more or less extensive pulmonary consolidation.

In the experiments referred to above, rather large doses of pneumococcus were used for infecting the animals, and it is probable that the controls were overwhelmed by the infection before there was an opportunity for pneumonia to develop. It is possible that if smaller doses had been employed for the intratracheal injections, Wadsworth would have produced pneumonia in the controls, and, on the other hand, the immunized animals would have escaped infection altogether.

The production experimentally of typical lobar pneumonia in monkeys affords an excellent method of testing the value of pneumococcus vaccine. It has been shown^{7,8} that pneumococcus pneumonia in monkeys differs in no respect clinically or pathologically from pneumococcus pneumonia in man. An inflammation of the lungs can be produced in rabbits, dogs, and other laboratory animals by introducing virulent pneumococci or streptococci into the trachea, but animals injected in this manner do not run the typical course of lobar pneumonia as observed in man. The object of the present study has been to determine first the value of prophylactic pneumococcus vaccination in general, and secondly, the relative merits of the different types of pneumococcus vaccine that have been employed.

⁶ Wadsworth, A., *Am. J. Med. Sc.*, 1904, cxxvii, 851.

⁷ Blake, F. G., and Cecil, R. L., *J. Exp. Med.*, 1920, xxxi, 403.

⁸ Blake, F. G., and Cecil, R. L., *J. Exp. Med.*, 1920, xxxi, 445.

Technique.

All the vaccine employed in this study was monovalent and was prepared from an old culture of *Pneumococcus* Type I which had been carried in the laboratory stock for several years. This organism appeared to have lost practically all its virulence. 1 cc. of a 24 hour broth culture had no effect whatever on a mouse when injected intraperitoneally. This was the same culture which was used at the Army Medical School in the preparation of pneumococcus vaccine for army camps.

The culture used for infecting the monkeys was a highly virulent *Pneumococcus* Type I, originally isolated from a case of lobar pneumonia. This organism killed a mouse in doses of 0.0000001 cc., and 0.00000001 cc. was usually lethal.

Method of Producing Experimental Pneumonia.—Experimental pneumonia was produced by introducing a small quantity of an 18 hour broth culture of pneumococcus (0.000001 to 1 cc.) with a Luer syringe directly into the trachea by the method previously described.⁷ Symptoms of pneumonia developed 24 to 48 hours after injection. In testing for resistance to infection following pneumococcus vaccination, the animals were injected intratracheally, in most cases 2 to 4 weeks after vaccination.

Experiments with Pneumococcus Type I Lipovaccine.

The first vaccine to be experimented with was a *Pneumococcus* Type I lipovaccine which had been prepared at the Army Medical School according to the process described by Whitmore, Fennel, and Petersen.⁹

18 hour glucose broth cultures of *Pneumococcus* Type I were centrifuged in a Sharpless machine. The sediment was dried at 53°C. for 24 hours. This killed all the pneumococci. The dried sediment was then weighed and ground with steel balls for 24 hours.

⁹ Whitmore, E. R., Fennel, E. A., and Petersen, W. F., *J. Am. Med. Assn.*, 1918, lxx, 427.

Finally, the dry powder was suspended in cottonseed oil containing 2 per cent lanolin and diluted to the desired strength.¹⁰

Dosage.—In order to make the results comparable with those in man only one injection of lipovaccine was administered to each monkey. As in man, the vaccine was injected subcutaneously, the abdominal wall being the site of inoculation.

Two series of monkeys were vaccinated; the first series received each the same dose that a man received of *Pneumococcus* Type I in the triple pneumococcus lipovaccine prepared by the Army Medical School; that is, 16 billion pneumococci, or 0.8 mg. of the dried bacteria. The second series received a dose proportional to their weight as compared with the weight of man. The average weight of a man is 70 kilos; the average weight of a Philippine monkey is 4 kilos. This series therefore received $\frac{1}{18}$ of man's dose, or 1 billion pneumococci (0.05 mg. of the dried bacteria). The lipovaccine was always diluted so that the monkey received 1 cc. of the oily suspension. In the first experiment a lipovaccine was used which was about 4 months old. In the remainder of the experiments, however, a freshly prepared vaccine was substituted.

Preliminary Test of Pneumococcus Lipovaccine.—The first attempt to test the efficacy of pneumococcus lipovaccine was carried out before the minimal infecting dose had been determined. As a result, the infecting dose which was employed in this experiment was 1 million times the size actually necessary to infect a normal monkey. The experiment is reported, however, as it illustrates certain differences between pneumonia in vaccinated and unvaccinated monkeys.

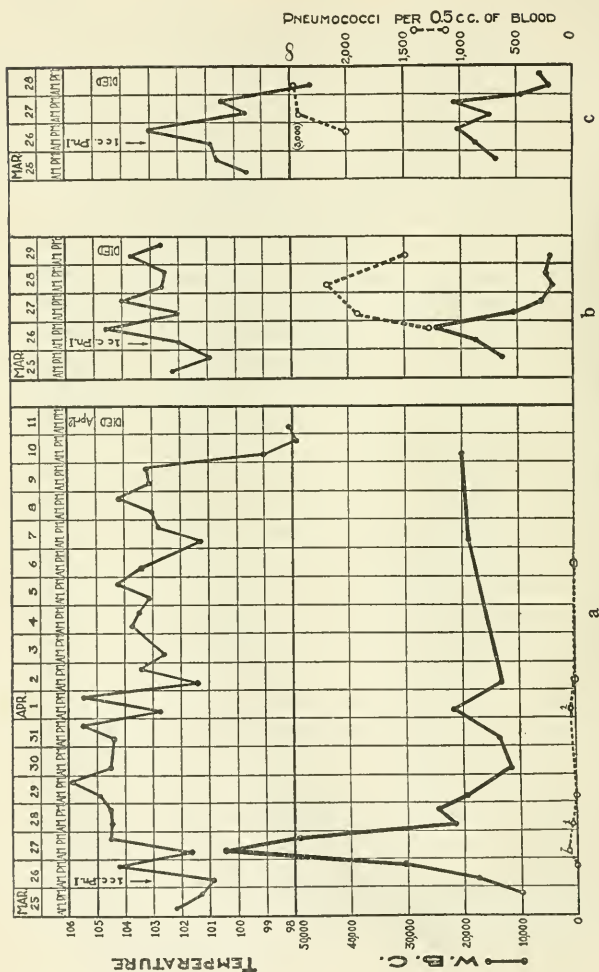
Experiment I.—Three *Macacus syrichtus* monkeys were used in this experiment (Table I). Monkey 14 had received a large dose (16 billion) of *Pneumococcus* Type I lipovaccine, Monkey 17 a small dose (1 billion) of the same, while Monkey 27 served as a control. On Mar. 26, 1919, each of these three monkeys received 1 cc. of an 18 hour broth culture of *Pneumococcus* Type I intratracheally.

The results are shown in Table I and Text-fig. 1. All three monkeys died; the vaccinated monkeys, however, lived longer than the control. Monkey 14, which

¹⁰ For full details for the method of preparing lipovaccine see articles by Whitmore, Fennel, and Petersen,⁹ Whitmore and Fennel (Whitmore, E. R., and Fennel, E. A., *J. Am. Med. Assn.*, 1918, lxx, 1902), and Fennel (Fennel, E. A., *J. Am. Med. Assn.*, 1918, lxxi, 2115).

TABLE I.
Experimental Pneumonia Following Vaccination.

Monkey No.	Weight.	Mar. 11. Fn. 1 lipovaccine subcutaneously.	Mar. 25. Serum tests.		Mar. 26. Broth culture of Fn. 1 intratracheally.	Result.	Autopsy.	Autopsy cultures.	
			Agglutins.	Protec- tion.				Lung.	Heart's blood.
14	<i>gms.</i> 2,850	0.8 mg. (16 bil- lion).	0	0	<i>cc.</i> 1	Died in 17 days.	Resolving lobar pneumo- nia; hypertrophy and di- latation of heart.	Sterile.	Pn. I
17	2,800	0.05 mg. (1 bil- lion).	0	0	1	" " 4 "	Lobar pneumonia; red stage.	Pn. I	" I
27	2,620	0	0	0	1	" " 3 "	Acute bronchitis; acute sup- purative pericarditis (Pn. I).	Sterile.	" I (bronchus, Pn. I).



TEXT-FIG. 1. *a*, *b*, and *c*. Pneumococcus Type I pneumonia in vaccinated monkeys following the intratracheal injection of a large dose (1 cc.) of Pneumococcus Type I culture. (*a*) Monkey 14; vaccinated on Mar. 11 with 0.8 mg. (16 billion) of Pneumococcus Type I lipovaccine subcutaneously. (*b*) Monkey 17; vaccinated on Mar. 11 with 0.05 mg. (1 billion) of Pneumococcus Type I lipovaccine subcutaneously. (*c*) Monkey 27; control.

received a large dose of vaccine, had apparently recovered on Apr. 10, but died suddenly on Apr. 12 from a greatly dilated heart. The leucocyte reactions were more marked in the vaccinated monkeys, though there was not a great deal of difference between Monkeys 17 and 27. Finally, the blood culture in Monkey 14, which received a large dose of vaccine, remained practically sterile, while in the other two monkeys the blood contained large numbers of pneumococci. The control monkey was overwhelmed by the huge infecting dose and died before frank pneumonia developed. He showed, in addition to hemorrhagic bronchitis, acute suppurative pericarditis.

In this experiment vaccination failed to protect either monkey against pneumonia, but the results in the case of one vaccinated monkey suggested that vaccination had modified to some extent the virulence of the infection.

Effect of Small Doses of Pneumococcus Lipovaccine.—If protection against pneumococcus infection in monkeys could be obtained by vaccination, it was desirable to find the minimum efficient dose; in other words, a dose that would be comparable with that used in man. In the following experiment the vaccinated monkeys had each received a dose of lipovaccine proportional to their weight as compared with the weight of a man; that is, 0.05 mg. of the dried bacteria, or 1 billion pneumococci.

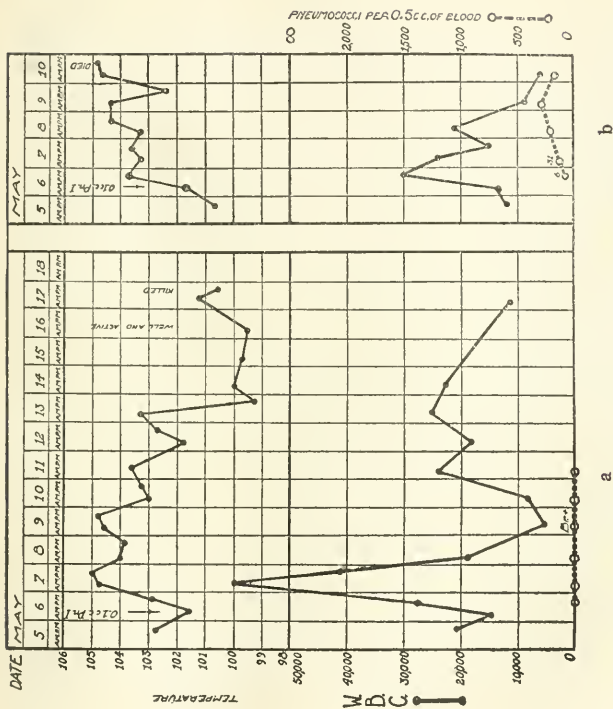
Experiment 2.—Six *Macacus syrichtus* monkeys were used in this experiment. Three (Monkeys 64, 65, and 67) had received 1 billion each of *Pneumococcus* Type I lipovaccine; the other three (Monkeys 85, 86, and 87) were controls (Table II). May 6, 1919. All six monkeys were injected intratracheally with an 18 hour broth culture of *Pneumococcus* Type I. Monkeys 65 and 87 received 0.1 cc.; Nos. 67 and 85 received 0.001 cc.; and Nos. 64 and 86 received 0.000001 cc.

All six monkeys promptly developed symptoms of pneumonia. It will be observed, however, that while the three control monkeys died, two of the vaccinated monkeys recovered. The third vaccinated monkey (No. 67) had a crisis on the 9th day, but died suddenly on the 11th day of the disease. Autopsy revealed an old aortic endocarditis and insufficiency, with cardiac hypertrophy and dilatation. The size of the dose did not appear to exert a very pronounced influence on the course of the disease in either vaccinated or unvaccinated monkeys. Table II shows the protocols of these experiments, and Text-figs. 2, 3, and 4 exhibit the temperature, leucocyte, and blood culture curves.

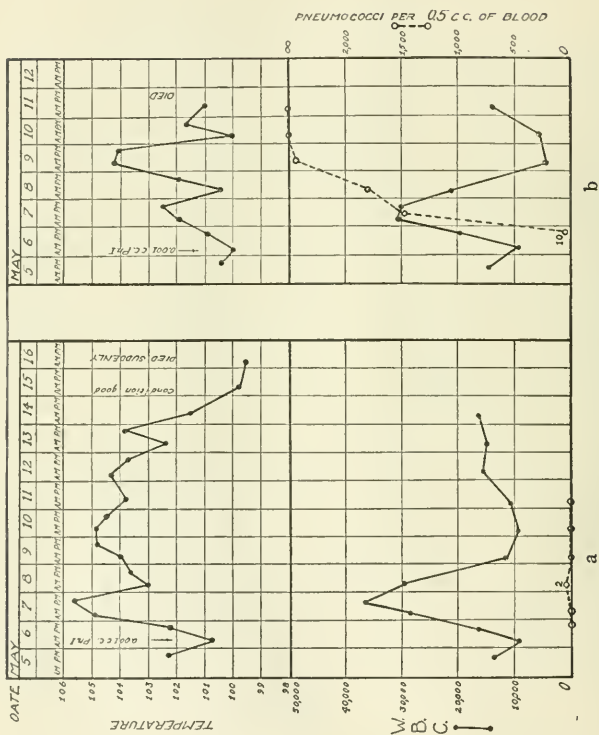
TABLE II.
Results of Vaccination with a Small Dose of *Pneumococcus Lipovaccinae*.

Monkey No.	Weight. gm.	Apr. 21. Pn. I lipovaccine subcutaneously.	May 6. Serum tests.		May 6. Broth culture of Pn. I intra- tracheally.	Result.	Autopsy.	Autopsy cultures.	
			Agglu- tination.	Protec- tion.				Lug.	Heart's blood.
65	3,075	0.05 mg. (1 bil- lion).	0	0	cc. 0.1	Clinical pneumonia. Recovery by crisis on 8th day. Killed on 12th day.	Resolving lobar pneu- monia, R. L.*	No growth.	No growth.
67	2,629	0.05 mg. (1 bil- lion).	0	0	0.001	Clinical pneumonia. Crisis on 9th day. Died on 11th day.	Resolving lobar pneu- monia; chronic aor- tic endocarditis and insufficiency; cardi- ac hypertrophy and dilatation.	Pn. I	Pn. I
64	2,599	0.05 mg. (1 bil- lion).	0	0	0.000001	Clinical pneumonia. Recovery by lysis on 26th day. Killed on 30th day.	Organizing lobar pneu- monia, L. L.	" I (4 col- onies).	No growth.
87	2,835	0			0.1	Clinical pneumonia. Died on 5th day.	Lobar pneumonia, L. U., L. M., L. L.; red stage.	Pn. I	Pn. I
85	2,675	0			0.001	Clinical pneumonia. Died on 6th day.	Lobar pneumonia, L. M., L. L.	" I	" I
86	2,424	0			0.000001	Clinical pneumonia. Died on 14th day.	Lobar pneumonia, R. L., L. L.; gray stage.	" I	No growth.

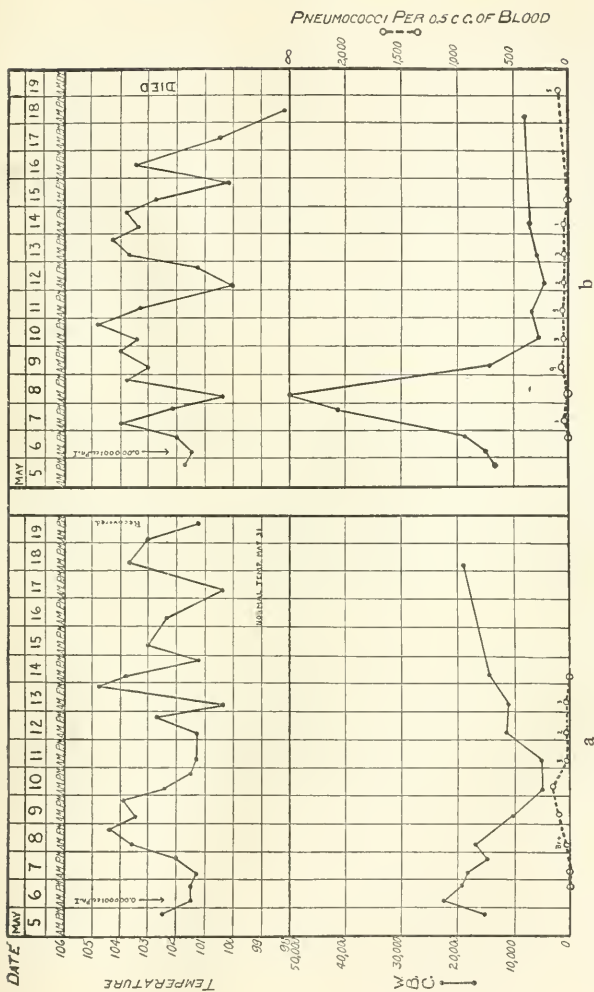
* L. U., L. M., L. L., etc., indicate lobes of the lung. The cardiac lobe is included as part of the right lower lobe.



TEXT-FIG. 2, *a* and *b*. *Pneumococcus* Type I pneumonia in a monkey vaccinated with a small dose (1 billion) of *Pneumococcus* Type I lipovaccine subcutaneously. (*a*) Monkey 65; vaccinated on Apr. 21. (*b*) Monkey 87; control.



TEXT-FIG. 3. *a* and *b*. Pneumococcus Type I pneumonia in a monkey vaccinated with a small dose (1 billion) of Pneumococcus Type I lipovaccine subcutaneously. (*a*) Monkey 67; vaccinated on Apr. 21. (*b*) Monkey 85; control.



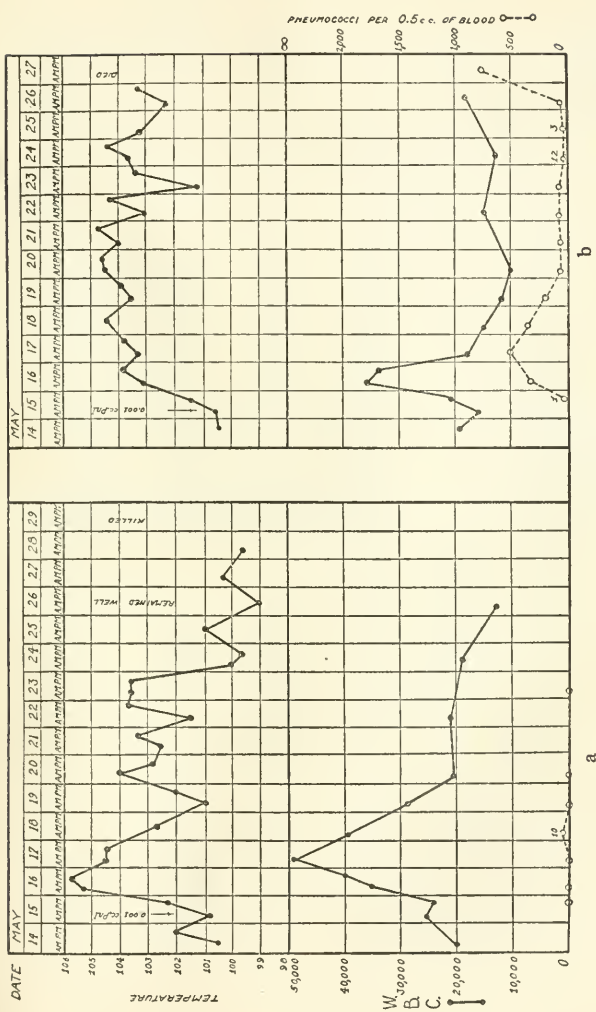
TEXT-FIG. 4. *a* and *b*. Pneumococcus Type I pneumonia in a monkey vaccinated with a small dose (1 billion) of Pneumococcus Type I lipovaccine subcutaneously. (*a*) Monkey 64; vaccinated on Apr. 21. (*b*) Monkey 86; control.

In these experiments, as in Experiment 1, it will be observed that the blood culture was sterile or weakly positive in vaccinated monkeys, while all the controls showed persistently positive blood cultures. These charts, however, demonstrate clearly that small doses of pneumococcus lipovaccine do not protect monkeys against pneumococcus pneumonia, even when the infecting dose is very small. It will also be observed that lipovaccine failed to stimulate agglutinins or protective substances in the monkeys' blood. Nevertheless, vaccination did appear to influence favorably the course of the disease.

Effect of Large Doses of Pneumococcus Lipovaccine.—In view of the failure of small doses of lipovaccine to protect against pneumonia, the next step was to determine the effect of large doses of pneumococcus lipovaccine. This experiment was carried out in the same manner as the one just described.

Experiment 3.—Six *Macacus syrichtus* monkeys were tested in this experiment (Table III, Text-figs. 5, 6, and 7). Monkeys 78, 80, and 81 had been vaccinated, each with 16 billion pneumococci. Monkeys 93, 95, and 96 were used for controls. All six monkeys were inoculated intratracheally with an 18 hour broth culture of *Pneumococcus* Type I. Monkeys 81 and 95 received 0.00001 cc., and Monkeys 80 and 96 received 0.000001 cc. on May 13, 1919. Monkeys 78 and 93 received 0.001 cc. on May 15. All six monkeys developed pneumonia. In this experiment two of the controls and two of the vaccinated monkeys recovered, while one in each series died. The death of the vaccinated monkey was unquestionably due to the complicating pericarditis which was discovered at autopsy.

As in Experiment 2, the vaccinated monkeys showed sterile or weakly positive blood cultures, with the exception of Monkey 80, in which the development of pericarditis probably contributed to the production of a fairly heavy blood infection. The character of the leucocyte reaction did not appear to be influenced by vaccination, nor was the disease appreciably shortened in the vaccinated group.



TEXT-FIG. 5. *a* and *b*. Pneumococcus Type I pneumonia in a monkey vaccinated with a large dose (16 billion) of Pneumococcus Type I lipovaccine subcutaneously. (*a*) Monkey 78; vaccinated on Apr. 29. (*b*) Monkey 93; control.

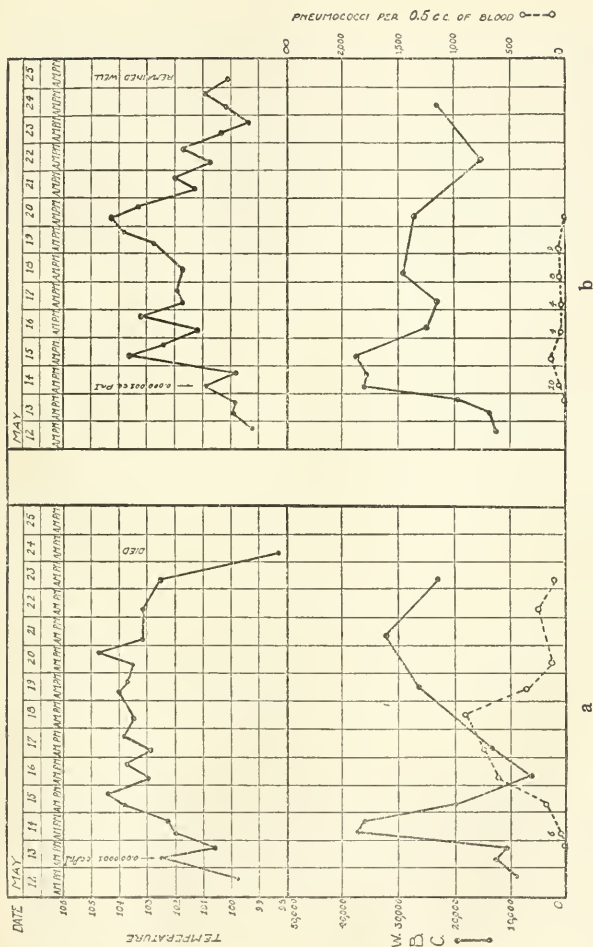
TABLE III.
Results of Vaccination with a Large Dose of *Pneumococcus Lipovaccine*.

Monkey No.	Weight. gm.	Apr. 29. Pn. I lipovaccine subcutaneously.	May 13. Serum tests.		May 13. Broth culture of Pn. I intra- tracheally.	May 15. Broth culture of Pn. I intra- tracheally.	Result.	Autopsy.	Autopsy cultures.		
			Agglutins.	Protec- tion.					Lung.	Heart's blood.	Pericar- dium.
78	5,322	0.8 mg. (16 bil- lion).	0	0	etc.	0.001	Clinical pneumonia. Recovery by cri- sis on 10th day. Killed on 15th day.	Resolving lobar pneumonia, R. L., L. M.	No growth.	No growth.	
81	2,750	0.8 mg. (16 bil- lion).	0	0	0.00001		Clinical pneumonia. Recovery by ly- sis on 10th day. Killed on 17th day.	Resolving lobar pneumonia, R. M., R. L.	"	"	
80	2,850	0.8 mg. (16 bil- lion).	0	0	0.000001		Clinical pneumonia. Died on 12th day.	Lobar pneumonia, L. L., L. M.; acute pericardi- tis.	" I	Pn. I	Pn. I
93	5,110	0				0.001	Clinical pneumonia. Died on 13th day.	Lobar pneumonia; gray stage; en- tire right lung.	" I	" I	

95	4,110	0	0.00001	Clinical pneumonia. Crisis on 11th day. Relapse. Recovery on 21st day. Killed on 21st day. Clinical pneumonia. Recovery by crisis on 8th day. Blood culture, Pn. I.	Lobar pneumonia, L. L.; resolution and organization.	No growth.	No growth.
96	2,655	0	0.000001				

TABLE IV.
Spontaneous Pneumonia Following Vaccination.

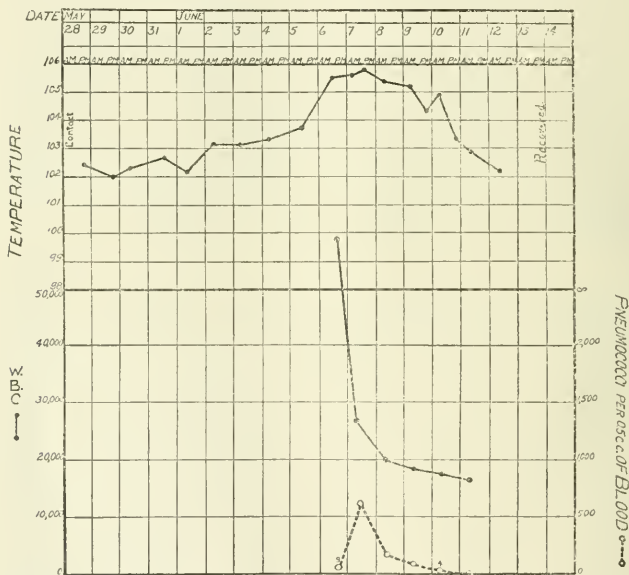
Monkey No.	Weight. gm.	Mar. 11. Pn. I lipovaccine subcutaneously.	Mar. 25. Serum tests with Pb. I.		Date of death.	Autopsy.	Autopsy cultures.		
			Agglutinos.	Protec- tion.			Lung.	Heart's blood.	Pericardium.
15	4,000	0.05 mg. (1 bil- lion).	0	0	1919 Apr. 9	Lobar pneumonia; stage of resolution.	Pn. IV	Sterile.	
13	5,055	0.05 mg. (1 bil- lion).	0	0	" 25	Lobar pneumonia; gray stage; acute pericarditis.	Sterile.	Pn. IV	Pn. IV
21	2,815	0.05 mg. (1 bil- lion).	0	0	" 29	Resolving lobar pneumonia; acute pericarditis.	"		Pn. (type unde- termined).



TEXT-FIG. 7. *a* and *b*. *Pneumococcus* Type I pneumonia in a monkey vaccinated with a large dose (16 billion) of *Pneumococcus* Type I lipovaccine subcutaneously. (*a*) Monkey 80; vaccinated on Apr. 29. (*b*) Monkey 96; control.

Contact Experiments.

From the preceding experiments it is evident that pneumococcus lipovaccine in the dosage employed failed to protect monkeys against experimental pneumonia. In order, however, to forestall the criticism which might be made that pneumonia had been produced by artificial means, it was decided to test the immunity of the vaccinated monkeys against spontaneous pneumonia by means of a contact test.



TEXT-FIG. 8. Spontaneous *Pneumococcus* Type I pneumonia developing in Monkey 16, vaccinated on Mar. 11 with a large dose (16 billion) of *Pneumococcus* Type I lipovaccine subcutaneously. Infection followed contact in the same cage with another case of *Pneumococcus* Type I pneumonia.

Experiment 4.—Three *Macacus syrichtus* monkeys which had previously been vaccinated with pneumococcus lipovaccine were placed in a cage with three normal healthy monkeys. Two monkeys in the active stage of *Pneumococcus*

Type I pneumonia were then put in the cage with the other six monkeys and the eight animals kept in intimate contact for 2 weeks. A few days after the experiment was started, Monkey 16, one of the vaccinated animals, became ill with pneumonia. The protocol follows:

Mar. 11, 1919. Monkey 16, *Macacus syrichtus*; weight 3,630 gm. Received 0.8 mg. (16 billion) of *Pneumococcus* Type I lipovaccine subcutaneously. May 26. Monkey placed in the same cage with two other vaccinated monkeys, three normal monkeys and two monkeys suffering with *Pneumococcus* Type I pneumonia. June 6. Monkey appears sick; marked leucocytosis. Blood culture shows *Pneumococcus* Type I. June 7. Typical lobar pneumonia; blood culture shows 650 colonies of *Pneumococcus* Type I per 0.5 cc. of blood. June 11. Marked improvement. Monkey has run a typical course of lobar pneumonia.

This experiment shows that Monkey 16, in spite of having been vaccinated with a large dose of pneumococcus lipovaccine, was unable to resist infection with *Pneumococcus* Type I when exposed to pneumonia due to this type. It will be observed, however, in Text-fig. 8, that the disease ran a mild and fairly short course, which supports the observation previously made that vaccinated monkeys tolerate pneumonia more readily than unvaccinated animals. Strangely enough, another one of the vaccinated animals in the contact test developed pneumonia, but in this instance the infection proved to be with *Pneumococcus* Type IV.¹¹ None of the control monkeys became infected.

Spontaneous Pneumococcus Type IV Pneumonia in Monkeys Vaccinated against Pneumococcus Type I.—Some of the monkeys that had been inoculated with *Pneumococcus* Type I lipovaccine were put back into a large cage with a number of stock monkeys. An epidemic of *Pneumococcus* Type IV pneumonia broke out in this cage and a number of the vaccinated monkeys contracted the disease.

Experiment 5.—Three *Macacus syrichtus* monkeys (Nos. 13, 15, and 21) had received a small dose of *Pneumococcus* Type I lipovaccine (Table IV). Pneumonia developed spontaneously in all of them 4 to 7 weeks after vaccination, just at the time when presumably their immunity should have been at a high point. In Monkeys 13 and 15 *Pneumococcus* Type IV was recovered from the autopsy cultures, and in Monkey 21 a pneumococcus was seen in the pericardial fluid, but failed to grow in the culture.

¹¹ See Paper I.⁷

The protocols demonstrate the fact that monkeys vaccinated with Pneumococcus Type I lipovaccine possess no demonstrable cross-immunity against spontaneous Pneumococcus Type IV pneumonia.

Summary of Lipovaccine Experiments.—A review of the experiments so far reported brings out these facts:

1. Pneumococcus Type I lipovaccine, whether used in large or small doses has failed to protect monkeys against experimental and spontaneous Pneumococcus Type I pneumonia.

2. Vaccination, however, does appear to modify the course of a subsequent Type I pneumonia. The blood is not so heavily infected as in unvaccinated animals, in some cases remaining practically sterile throughout the entire course of the disease. Furthermore, the mortality rate is lower in the vaccinated monkeys and the disease seems to run a milder course.

3. No agglutinins or protective bodies were demonstrated in any of the monkeys inoculated with lipovaccine.

4. There is no evidence that Pneumococcus Type I lipovaccine confers any cross-immunity against other types of pneumococcus pneumonia.

Experiments with Pneumococcus Type I Saline Vaccine.

The failure of pneumococcus lipovaccine to protect monkeys against pneumonia prompted us to test the value of pneumococcus saline vaccine. Such experiments seemed all the more justified in view of the fact that the results of prophylactic vaccination against pneumonia at Camp Upton, where a saline vaccine had been used, were distinctly better than the results at Camp Wheeler where the lipovaccine had been employed.

The pneumococcus saline vaccine was prepared from the same avirulent strain of Pneumococcus Type I which had been used in the preparation of the Pneumococcus Type I lipovaccine. The saline vaccine was made as follows:

Pneumococci were cultivated for 18 hours in glucose broth and submitted to centrifugation. The bacterial sediment was then heated at 55°C. for 1 hour to kill the pneumococci. The vaccine was diluted with normal salt solution containing 0.25 per cent tricresol

and standardized by Wright's method. The saline vaccine used in the following experiments was prepared on May 1, and the experiments were started on May 6, 1919.

For the most part, the dosage and method of administration in the experiments with pneumococcus saline vaccine were the same as in the lipovaccine tests. In the following experiments each monkey received only one subcutaneous injection.

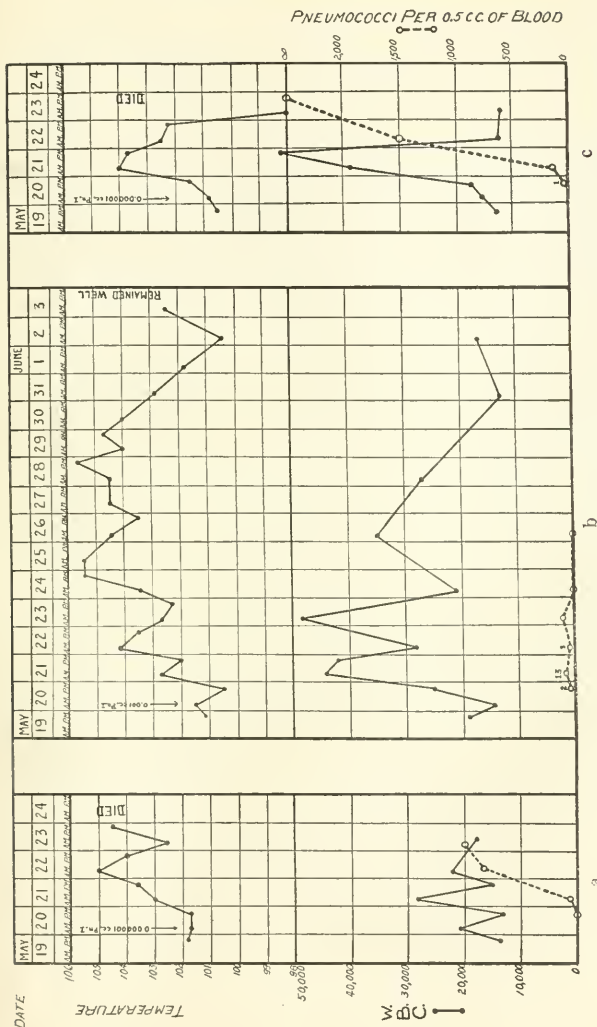
Results of Vaccination with Pneumococcus Type I Saline Vaccine.—In testing the saline vaccine the effect of the large and small dosage was determined in one experiment.

Experiment 6.—Four *Macacus syrichtus* monkeys were used in this experiment (Table V, Text-figs. 9 and 10). Monkeys 88 and 89 had each been vaccinated with 1 billion, Monkey 90 with 16 billion *Pneumococcus* Typé I saline vaccine. Monkey 98 was the control. 2 weeks after these monkeys were vaccinated, their blood was tested for agglutinins and protective bodies. No agglutinins could be demonstrated, but all three monkeys showed the presence of protective bodies. In Monkeys 89 and 90 the protection was marked, in Monkey 88 slight. 2 weeks after vaccination the monkeys were injected intratracheally with an 18 hour broth culture of *Pneumococcus* Type I. Monkeys 88, 90, and 98 received each 0.000001 cc. of culture. Monkey 89 received 0.001 cc. of culture. The results are shown in Table V. While the four monkeys all developed pneumonia, the control monkey ran a rapid course and died on the 4th day. The vaccinated animals lived longer and two of them recovered (Monkeys 89 and 90). Monkey 88 died on the 5th day. The two cases that terminated fatally showed extensive lobar pneumonia at autopsy and *Pneumococcus* Type I was recovered from the lungs and heart's blood. One of the vaccinated monkeys that recovered was killed and at autopsy showed a resolving pneumonia, cultures from which were sterile. The temperature, leucocyte, and blood culture curves are shown in Text-figs. 9 and 10.

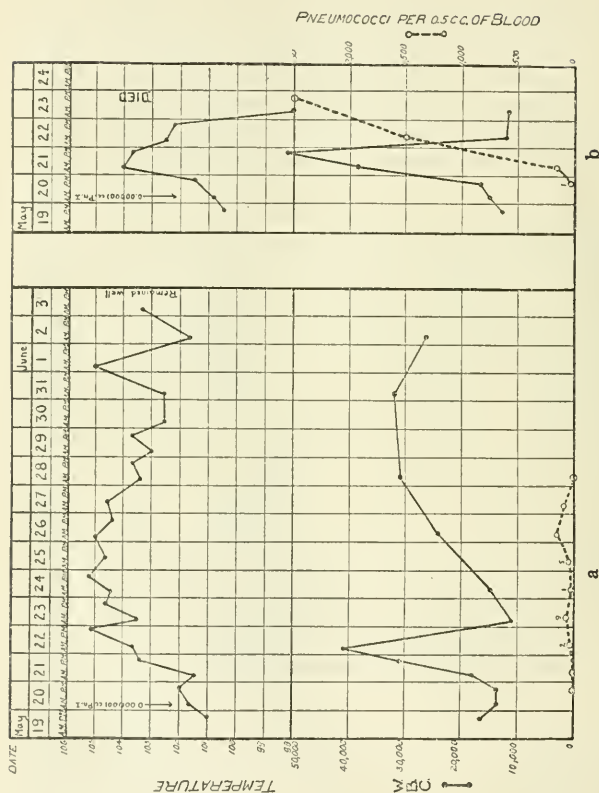
With pneumococcus saline vaccine as with lipovaccine, prophylactic inoculation failed to protect monkeys against pneumonia, but, as in the case of lipovaccine, inoculation seemed to modify favorably the course of the disease. The vaccinated monkey that died (Monkey 88) was the one which showed the smallest amount of protective substances in its blood. The two vaccinated monkeys which recovered showed only a moderate degree of bacteremia, whereas the two monkeys that died had heavy blood infections. The amount of pneumococcus culture used for infecting the monkeys appears to have

TABLE V.
Results of Vaccination with *Pneumococcus Saline Vaccine*.

Monkey No.	Weight gm.	May 6. Pn. I saline vaccine sub- cutaneously.	Agglut. mins.	May 19. Serum tests.		May 20. Broth culture of Pn. I intra- tracheally.	Result.	Autopsy.	Autopsy cultures.	
				Protection.	Control.				Lung.	Heart's blood.
88	2,072	1 billion.	0	0.00001 cc. Died in 60 hrs. 0.000001 cc. Died in 60 hrs. 0.0000001 cc. Survived.	0.00001 cc. Died in 24 hrs. 0.000001 cc. Died in 48 hrs. 0.0000001 cc. Died in 48 hrs.	cc. 0.0000001	Clinical pneumo- nia. Died on 5th day.	Lobar pneumo- nia, entire left lung, R. L.; red stage.	Pn. I	Pn. I
89	2,462	1 "	0	0.00001 cc. Sur- vived. 0.000001 cc. Sur- vived. 0.0000001 cc. Survived.		0.001	Clinical pneumo- nia. Recovery by lysis on 12th day.	Resolving lobar pneumonia, R. L.	No growth.	No growth.
90	3,922	16 "	0	0.00001 cc. Sur- vived. 0.000001 cc. Sur- vived. 0.0000001 cc. Survived.		0.0000001	Clinical pneumo- nia. Recovery by lysis on 11th day.			
98	4,100	0		0.000001 cc. Sur- vived. 0.0000001 cc. Survived.		0.0000001	Clinical pneumo- nia. Died on 4th day.	Lobar pneumo- nia, R. U., R. L., L. U., L. L.; stage of en- gorgement.	Pn. I	Pn. I



TEXT-FIG. 9. *a*, *b*, and *c*. Pneumococcus Type I pneumonia in monkeys vaccinated with a small dose (1 billion) of Pneumococcus Type I saline vaccine subcutaneously. (*a*) Monkey 88; vaccinated on May 6. (*b*) Monkey 89; vaccinated on May 6. (*c*) Monkey 98; control.



TEXT-FIG. 10, *a* and *b*. *Pneumococcus* Type I pneumonia in a monkey vaccinated with a large dose (16 billion) of *Pneumococcus* Type I saline vaccine subcutaneously. (*a*) Monkey 90; vaccinated on May 6. (*b*) Monkey 98; control.

little influence, up to a certain point, upon the course of the disease. Monkey 88 received only 0.000001 cc. and died on the 5th day. Monkey 89 received 0.001 cc. (a thousand times as large a dose) and recovered from the infection after running a comparatively mild course.

Results of Vaccination with Three Injections of Pneumococcus Type I Saline Vaccine.—Although one injection of pneumococcus saline vaccine failed to protect monkeys against pneumococcus infection, the results were rather more encouraging than those obtained with the lipovaccine. In two of the vaccinated monkeys tested, a high degree of protection was demonstrable in the blood following the inoculation, and although these two monkeys both contracted pneumonia, the disease ran a mild course and both monkeys recovered. Therefore it seemed desirable to determine whether three injections of pneumococcus saline vaccine given at intervals of 1 week would not afford the necessary amount of protection.

Experiment 7.—This series of *Macacus syrichtus* monkeys was started on saline vaccine May 22, 1919, each monkey receiving weekly subcutaneous injections of 1 billion pneumococci until three inoculations had been given. 2 weeks after the third injection the blood from these monkeys was tested for agglutinins and protective bodies. No agglutinins or protective bodies could be demonstrated in any of the five monkeys tested.

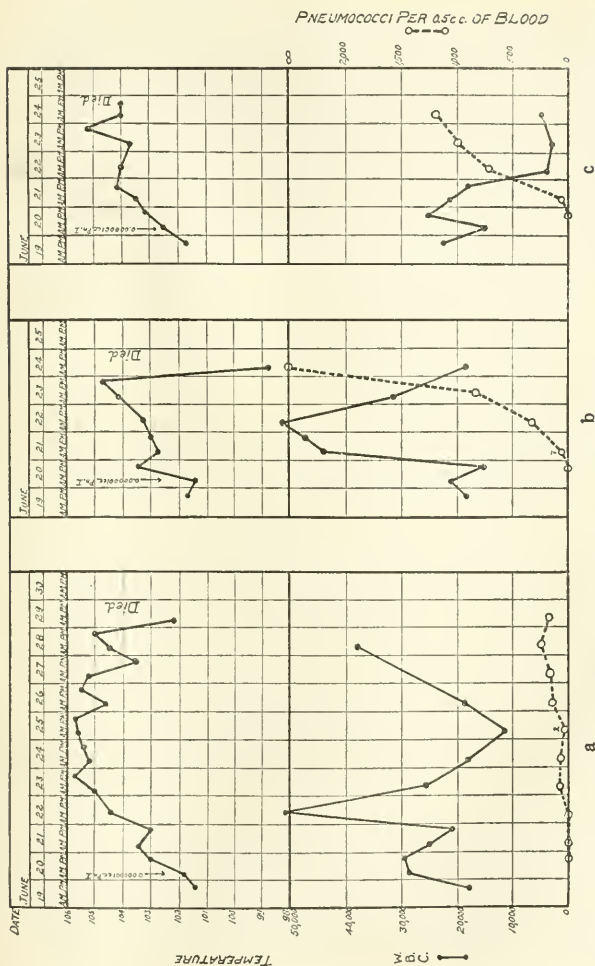
June 20. Two of the vaccinated monkeys and a control monkey were injected intratracheally with 0.000001 cc. of an 18 hour broth culture of *Pneumococcus* Type I. Table VI and Text-fig. 11 show the results obtained. All three monkeys developed pneumonia, and in all three the disease was fatal. In one vaccinated monkey (Monkey 100) the disease presented features which are usually associated with a mild attack; namely, a moderate infection of the blood and a good secondary rise in the leucocytes, while in the other (Monkey 101) a septicemia equally as heavy as that of the control developed. At autopsy all three monkeys showed lobar pneumonia, and *Pneumococcus* Type I was recovered from the organs.

In this experiment three small doses of saline vaccine failed to give as much protection as had been obtained by a large single injection in the previous experiments.

Summary of Saline Vaccine Experiments.—The experiments which have been reported indicate that saline vaccine like lipovaccine, when injected subcutaneously in moderate doses, has failed to protect

TABLE VI.
Results of Vaccination with Three Injections of Pneumococcus Saline Vaccine.

Monkey No.	Weight.	Pn. I saline vaccine subcutaneously.			June 20. Serum tests.		June 20. Growth culture of Pn. I intra- tracheally.	Result.	Autopsy.	Autopsy cultures.	
		May 22.	May 29.	June 5.	Agglutination.	Proteolysis.				Lung.	Heart's blood.
100	2,302 gm.	1 billion.	1 billion.	1 billion.	0	0	cc. 0.000001	Clinical pneumonia. Died on 10th day.	Lobar pneumonia, L. U., L. M., L. L., R. L.; gray stage.	No growth.	Pn. I
101	2,532	1 " "	1 " "	1 " "	0	0	0.000001	Clinical pneumonia. Died on 5th day.	Lobar pneumonia, L. U., L. M., L. L., R. L.; stage of en- gorgement.	Pn. I	" I
114 (control)	3,000	0	0	0			0.000001	Clinical pneumonia. Died on 5th day.	Lobar pneumonia, L. L., R. L.; stage of engorgement.	" I; S. hemolyticus.	" I; S. hemolyticus.



TEXT-FIG. 11, a, b, and c. Pneumococcus Type I pneumonia in monkeys vaccinated with three injections (1 billion each) of Pneumococcus Type I saline vaccine subcutaneously. (a) Monkey 100. (b) Monkey 101. Both these animals received vaccine on May 22, 29, and June 5. (c) Monkey 114; control.

monkeys against pneumococcus pneumonia. As for any relative superiority of one vaccine over the other, there appears to be little choice between the two. Saline vaccine, however, is more likely to stimulate the formation of protective bodies in the blood, and for this reason probably gives a somewhat better immunity. The individual variation in the natural resistance of monkeys to pneumococcus infection is a factor of primary importance and one which must always be considered. No definite decision can be reached as to the relative merits of pneumococcus lipovaccine and saline vaccine, except by testing a large series of monkeys with each type.

Effect of Intravenous Injection of Living Pneumococcus Type I Cultures in Monkeys Vaccinated with Pneumococcus Type I Vaccine.

In the preceding experiments it has been shown that pneumococcus vaccine does not protect monkeys against intratracheal infection with pneumococcus. It seemed desirable, therefore, for the sake of comparison, to determine whether these vaccinated monkeys would be protected against intravenous infection.

Experiment 8.—July 8, 1919. Two *Macacus syrichtus* monkeys that had been vaccinated 4 weeks previously with three injections (1 billion each) of Pneumococcus Type I saline vaccine and one control monkey were injected intravenously with 0.001 cc. of a broth culture of living virulent Pneumococcus Type I. This dose is often fatal for a normal monkey. The results are shown in Table VII and Text-fig. 12. The vaccinated monkeys showed few or no clinical symptoms following the injection. Monkey 105 remained perfectly well, with sterile blood cultures. Monkey 104, the other vaccinated monkey, had a mild febrile reaction and a temporary infection of the blood of 48 hours duration. The control (Monkey 116) was ill for 6 days with high fever and heavy septicemia.

All three monkeys were killed just after their temperature had returned to normal, and all showed perfectly normal lungs.

This experiment has considerable significance in suggesting that distinction must be made between a humoral immunity against pneumococcus and a local immunity, possibly cellular, in the lungs. The same dose of culture which Monkeys 104 and 105 received intravenously with impunity would have produced a severe pneumonia if administered intratracheally. Although this phenomenon may at

TABLE VII.
Effect of Intravenous Injection of Virulent Pneumococcus Type I in Monkeys Previously Vaccinated with Pneumococcus Type I Saline Vaccine.

Monkey No.	Weight. gm.	Pn. I saline vaccine subcutaneously.			June 21. Serum tests.		July 8. Broth culture of Pn. I intra- venously.	Result.	Autopsy.	Autopsy cultures. Heart's blood.
		May 22.	May 29.	June 5.	Agglut- ins.	Protec- tion.				
105	1,375	1 billion.	1 billion.	1 billion.	0	0	cc. 0.001	No clinical symptoms; no sep- ticemia. Killed on 5th day.	Normal lungs.	No growth.
104	1,735	1 "	1 "	1 "	0	0	0.001	Fever for 2 days; mild septi- cemia for 2 days; no symp- toms of pneumonia. Killed on 7th day.	" "	" pneumo- cocci.
116 (control).	1,935	0	0	0			0.001	Fever for 6 days; heavy septi- cemia; no symptoms of pneu- monia. Killed on 8th day.	" "	No growth.

first seem paradoxical, it is in reality not out of harmony with the results obtained in the preceding experiments in which it has been clearly shown that prophylactic vaccination prevented to a large extent the development of septicemia during the course of lobar pneumonia. It furthermore demonstrates that tests of the prophylactic value of vaccination in animals may lead to false conclusions if these depend upon the demonstration of immunity to intravenous infection rather than to the actual disease against which the vaccination is directed.

DISCUSSION.

In this study all efforts to protect monkeys against pneumonia by subcutaneous vaccination with killed cultures of pneumococcus have failed. With our present ignorance concerning the nature of bacterial antigen, any investigation of this nature must necessarily be for the most part empirical. In the experiments reported, two kinds of vaccine have been tried—the oily and the saline. But obviously, many other factors determine the character of a vaccine, each of which should be considered in determining the value of the vaccine for prophylactic inoculation. Among these factors are the following:

Virulence of the Organism.—It has generally been assumed that a virulent organism produces a more efficient vaccine than an avirulent strain. The virulent strain, however, causes a more severe reaction, and for this reason most vaccines in general use are prepared from avirulent cultures. The question of whether a vaccine prepared from a highly virulent pneumococcus will afford better protection than one prepared from an avirulent strain has not been attacked in the present study. It may, however, be a more important factor than is generally assumed and it is hoped that this question can be investigated at a later time.

Method of Cultivation.—When vaccines are prepared from cultures grown on solid media, all the products of bacterial metabolism are presumably included in the vaccine. When, however, the bacteria are grown in liquid media, the supernatant fluid is discarded, and with it the bacterial metabolites. Just how much antigenic value these products have is problematical. It is obvious, moreover, that in vaccines prepared from broth media, the longer the period of incu-

bation, the greater the autolysis and the greater the amount of bacterial products in solution in the broth. The influence of sugars, animal sera, etc., when added to the culture medium may be an important factor in the quality of a vaccine.

Method of Killing the Bacteria.—Many methods have been used for killing the bacteria in vaccines, but heat and germicides remain the two most frequently employed. In the case of the lipovaccine and saline vaccine used in the present study the pneumococci were killed by heat; but in the lipovaccine the bacteria were heated for 24 hours at 53°C., whereas in the preparation of the saline vaccine they were heated for only 1 hour at 55°C. Whether this difference in the duration of heating had any effect on the antigenic value of the vaccine, it is impossible to say.

Vehicle in Which the Bacteria Are Suspended.—Until quite recently all vaccines were prepared in either water or normal salt solution. The employment of vegetable oils as a vehicle for suspending bacteria introduces a new factor into the question of antigenic value. It is possible that in oily vaccines a capsule forms around the body of the microorganism and interferes not only with its absorption but also with the production of specific antibodies. In these experiments monkeys injected with pneumococcus lipovaccine failed to develop demonstrable agglutinins and protective bodies.

Age of the Vaccine.—The age of the vaccine is undoubtedly an important factor in its antigenic value and one that has not been thoroughly investigated. The lipovaccine used in the first of these experiments was 4 months old at the time the experiment was started. In the later experiments a fresh lipovaccine was used, prepared in the same way as the older one, and no difference in effect was observed in the two vaccines. With the saline vaccine, the first experiment, which was carried out immediately after the vaccine was prepared, showed protective bodies in the three monkeys tested. A month later the same vaccine was used for testing the effect of three repeated injections of saline vaccine and no protective bodies could be demonstrated in any of the five monkeys vaccinated. Whether this was a matter of individual variation in the monkeys or whether there occurred certain changes in the vaccine due to standing 1 month in the ice box, it is hard to say. The former hypothesis would appear more reasonable.

The results obtained in this study of prophylactic vaccination against pneumonia in monkeys have been disappointing; but it should be borne in mind that the test applied has been a particularly crucial one. Comparatively small doses of vaccine have been used in order to make the results comparable with vaccination in man. No doubt a satisfactory immunity could have been obtained if repeated injections of large doses of vaccine had been administered, and still better results might have been reached if, in the case of saline vaccine, the injections had been given intravenously. Such an accomplishment, however, was not the aim of the investigation.

Furthermore, the Type I pneumococcus which was employed in the intratracheal injections was an organism of extraordinary virulence. It practically never failed to produce the disease even in doses of 0.000001 cc. of broth culture, and in unvaccinated monkeys the result was usually fatal. One hundred millionth of a cc. was in most cases lethal for a mouse.

Finally, it must be emphasized that the monkey is highly susceptible to the pneumococcus. The prevalence of respiratory infections among these animals is well known; and that they succumb readily to the pneumococcus is evidenced by the fact that an epidemic of *Pneumococcus* Type IV pneumonia broke out among our stock monkeys and killed between 30 and 40 of them in less than 4 weeks. The disease ran through these animals, fresh from the Tropics, in very much the same manner that measles and pneumonia ravaged our southern recruits in 1917 and 1918. In either instance it was a case where an organism was suddenly brought in contact with a disease to which it had not been previously exposed. These monkeys when living in their natural environment probably rarely encountered the pneumococcus and had acquired no racial immunity to pneumococcus infections. Man, on the other hand, at least in North America, and particularly in urban communities, is constantly exposed to pneumococcus infections, and by reason of this exposure has probably gradually built up a fair degree of immunity against the microorganism. Clough¹² has recently shown that 19 per cent of normal men have demonstrable protective substances against pneu-

¹² Clough, P. W., *J. Am. Med. Assn.*, 1919, lxxiii, 785.

mococcus in their serum. Lack of previous exposure to pneumococcus is evidenced in monkeys by the difficulty which one has in producing protective substances in their blood.

A close analogy exists in this connection between pneumonia and typhoid fever. Metchnikoff and Besredka¹³ in their study of experimental typhoid fever found that it was impossible to protect apes against the disease by means of killed cultures. Most of the typhoid vaccine, however, used in this country and elsewhere has been composed of killed bacilli and the results obtained with this vaccine are sufficient justification for its further use. To reason too closely, therefore, from monkey to man may lead to false conclusions. The bearing of this discussion on the question of prophylactic vaccination against pneumonia in man is obvious. The value of such vaccination will have to be finally determined by vaccinating large groups of men living under approximately the same conditions, and the results controlled by observations upon similar unvaccinated groups.

Pneumococcus vaccine probably stimulates in every case the production of a certain quantity of antibody, an amount, however, which in monkeys is not sufficient to protect them against pneumonia. Usually the antibody production in monkeys is not of sufficient degree to be demonstrable by any laboratory test. It is sufficient, however, to modify the course of the disease. The bacteremia is distinctly less marked. In twelve vaccinated monkeys the mortality rate was 41.6 per cent, while for seventeen unvaccinated monkeys the mortality rate was 76.4 per cent. Other evidence for this antibody production is furnished by the resistance which vaccinated monkeys offer to infection by the intravenous route.

In conclusion, it must be emphasized that immunity is a purely relative term. Almost any animal's "immunity," so called, can be overcome by a sufficiently large injection of virulent bacteria.

¹³ Metchnikoff, E., and Besredka, A., *Ann. Inst. Pasteur*, 1911, xxv. 931; 1913, xxvii, 597.

CONCLUSIONS.

1. The subcutaneous inoculation of monkeys with *Pneumococcus* Type I vaccine in doses comparable with those employed in man does not protect them against subsequent attacks of *Pneumococcus* Type I pneumonia, either spontaneous or experimental. Furthermore, the occurrence of *Pneumococcus* Type IV pneumonia among monkeys that have been vaccinated with *Pneumococcus* Type I lipovaccine indicates that the vaccinated animals develop no cross-protection against other types of pneumonia.

2. Vaccination does, however, modify the course of the disease. Invasion of the blood stream by the pneumococcus in vaccinated animals is usually slight, and the proportion of recoveries is considerably higher for vaccinated than for unvaccinated monkeys.

3. *Pneumococcus* saline vaccine produces a greater amount of protective substance in the serum of the vaccinated animal than does pneumococcus lipovaccine and is probably, therefore, a better antigen. Both, however, fail to protect the animal against pneumococcus pneumonia.

4. Subcutaneous vaccination with pneumococcus vaccine gives definite protection against experimental pneumococcus septicemia. In other words, vaccination may induce a humoral immunity without protecting against intratracheal infection.

5. In view of the fact that monkeys are highly susceptible to pneumococcus infection, a strict analogy cannot be drawn between pneumococcus immunity in monkeys and pneumococcus immunity in man, since in the latter a considerable amount of resistance already exists, probably by reason of repeated exposure to pneumococcus infection.

EXPERIMENTAL STUDIES ON DIABETES.

SERIES I. PRODUCTION AND CONTROL OF DIABETES IN THE DOG. 3. EFFECTS OF PROTEIN DIETS.

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Comparison of Carbohydrate and Protein.

In mildly diabetic dogs the starch of 50 to 100 gm. of bread or cereal is more active in producing glycosuria than the larger quantity of potential carbohydrate represented in a kilo of beef lung.¹ Undoubtedly the starch would be much better assimilated if distributed in small doses over a longer time, but the supposition that the difference is only one of rapidity of absorption and metabolism is opposed by the following facts: (1) The glycosuria from starch in these animals generally lasts over 6 or even 12 hours, while phlorizin and respiration experiments prove that protein requires no longer time for absorption and deamination. (2) The effect of carbohydrate is often cumulative. For example, Dog B2-89 was free from glycosuria on 1 kilo of beef lung daily. On June 25, 50 gm. of bread were substituted for 250 gm. of the lung. The urine remained negative till a glycosuria of 0.75 per cent appeared on June 30 and 0.71 per cent on July 1. Then on return to the diet of 1 kilo of lung, the urine remained negative up to the following test in August. On August 5, 25 gm. of bread were substituted for 150 gm. of lung. A trace of glycosuria appeared on August 10, and 0.18 per cent on August 14. On August 15, nothing was fed but 50 gm. of bread, and an excretion of 1.4 per cent sugar in 140 cc. of urine resulted. On resumption of the diet of 1 kilo of lung glycosuria remained absent up to the time of another experi-

¹ Allen, F. M., *J. Exp. Med.*, 1920, xxxi, 397-399.

ment on September 17. (3) Blood sugar analyses to be reported later show that protein causes little hyperglycemia in mild diabetes, but in severe diabetes it produces a blood sugar curve resembling that of carbohydrate in milder cases, not only in height but also in rapidity of rise, thus confirming the statement (1) above concerning time relations.² Also the maximal dextrose-nitrogen ratios in the severest diabetes are a familiar proof that the carbohydrate of protein is excreted quantitatively and is as incapable of assimilation as preformed carbohydrate.

These facts conform to clinical observations and justify the earlier treatment of diabetes, from the time of Rollo onward, inasmuch as the restriction of preformed carbohydrate is after all the foundation stone of dietotherapy, and protein restriction comes secondary in both time and importance.

This simple point also has relation to the question of whether diabetes is a deficiency of utilization of glucose primarily and specifically or of other foods as well. The progressive impairment of protein metabolism, from the stage where protein is apparently assimilated almost perfectly to the stage where it causes hyperglycemia and glycosuria almost identical with those from preformed carbohydrate, has an important bearing upon this question but does not in itself furnish a decisive answer.

Comparisons of Proteins.

Dog B2-29.—This dog, having severe diabetes and a very low tolerance, with a remnant of approximately $\frac{1}{4}$ of the pancreas, was used for feeding tests with approximately 10 gm. of protein in different forms (Table I).

Tests with different forms of protein in other animals gave similar results. Also Dog B2-25, possessing $\frac{1}{12}$ — $\frac{1}{13}$ of the pancreas, was used for a more thorough test of pancreas feeding. The body weight was 11.3 kilos on July 4, 1914; 500 gm. of fresh raw beef pancreas were fed daily until July 18, then 600 gm. daily till July 28, then 750 gm. of pancreas till Aug. 2, when the body weight was 14.4 kilos. Slight glycosuria was present from July 30 to Aug. 2. Fasting from Aug. 2 to 10 reduced the body weight to 12.5 kilos. A diet of 500 gm. of beef lung was then given daily to Aug. 18, 600 gm. daily to Aug. 27, 750 gm. daily to Sept. 28,

² Cf. one example already reported, Allen F. M., *Am. J. Med. Sc.*, 1917, cliii, 362, Chart 8, Dog 386.

800 gm. daily to Oct. 10, 1,000 gm. daily to Oct. 17, 1,200 gm. daily to Oct. 21, and 1,400 gm. daily to Nov. 28. Up to Oct. 29, the lung was boiled after weighing; after that date it was fed raw. Glycosuria was absent till Nov. 25, when 4.1 gm. of sugar were excreted, increasing to 8.2 gm. on Nov. 27. The body weight at this time was 17 kilos. The reason for the higher assimilation of beef lung at a higher body weight was that the dog was gaining tolerance with time. Evidently there is nothing in raw pancreas which alters the natural customary progress of this improvement.

TABLE I.
Dog B2-29.

Date.	Diet.	Glycosuria.
1913		gm.
Dec. 29	50 gm. of raw beef.	0
" 31	30 " " soy beans (boiled).	0.2
1914		
Jan. 1	70 " " egg (approximately 2 small eggs), raw.	Trace.
" 2	100 " " lard (no protein).	0
" 3	50 " " raw pancreas.	0.16

Several experiments for comparison of raw and cooked meat (ordinary beef for boiling or beef lung) are typified by the following example.

Dog D4-74.—Female; mongrel; age 4 years; moderately well nourished. Jan. 17, 1917. Partial pancreatectomy leaving remnant estimated at $\frac{1}{4}$. Weight throughout feeding experiments 13.2 to 13.5 kilos. The quantities of beef lung mentioned (Table II) were either boiled after weighing or fed raw.

The literature to which these experiments are related has been reviewed previously.³ It was confirmed in numerous observations⁴ that after a period of carbohydrate-free diet or especially of fasting the feeding of carbohydrate causes glycosuria, which ceases on continuance of the same carbohydrate diet. Apparently some state of unpreparedness of the body for the sudden flood of carbohydrate is

³ Allen, F. M., Studies concerning glycosuria and diabetes, Cambridge, 1913, 442, 531, 813-815.

⁴ Cf. Dog B2-63, Mar. 30 and Dec. 16, 1915 (Allen,¹ p. 387); also Paper 4 of this series (*J. Exp. Med.*, 1920, xxxi, 576).

TABLE II.

Dog D-74.

Date.	Diet.		Glycosuria.
<i>1917</i>			<i>gm.</i>
Apr. 16	400 gm. of cooked lung;	100 gm. of suet.	0
" 17	500 " " " "	100 " " "	0
" 18	600 " " " "	100 " " "	0
" 19	600 " " " "		0
" 20	600 " " raw "		0
" 21	700 " " " "		0
" 22	700 " " cooked "		0
" 23	800 " " " "		0
" 24	800 " " " "		0.8
" 25	800 " " raw "		Faint.
" 26	800 " " cooked "		"
" 27	800 " " raw "		0
" 28	900 " " cooked "		Very faint.
" 29	900 " " raw "		0.25
" 30	900 " " cooked "		Faint.
May 1	1,000 " " raw "		Slight.
" 2	1,000 " " cooked "		Faint.
" 3	1,000 " " " "		Very faint.
" 4	1,000 " " " "	25 gm. of bread.	10.9
" 5	1,000 " " raw "	25 " " "	6.7
" 6	1,000 " " " "		5.1
" 7	1,000 " " " "		Slight.
" 8	1,000 " " cooked "		0.5
" 9	1,000 " " raw "		2.2
" 10	1,000 " " cooked "		Faint.
" 11	1,000 " " raw "		1.9
" 12	1,000 " " cooked "		Faint.
" 13	1,000 " " raw "		"
" 14	1,000 " " " "		0
" 15	1,000 " " cooked "	25 gm. of bread.	Faint.
" 16	1,000 " " raw "	25 " " "	3.7
" 17	1,000 " " " "		Faint.
" 18	1,000 " " cooked "	25 gm. of bread.	3.6
" 19	1,000 " " raw "	25 " " "	6.3
" 20	1,000 " " cooked "	25 " " "	0
" 21	1,000 " " raw "	25 " " "	5.7
" 22	1,000 " " cooked "	25 " " "	8.2
" 23	1,000 " " raw "	25 " " "	2.9
" 24	1,000 " " cooked "	25 " " "	3.4
" 25	1,000 " " raw "	25 " " "	4.1
" 26	1,000 " " cooked "	25 " " "	0
" 27	1,000 " " raw "	25 " " "	2.7
" 28	1,000 " " cooked "	25 " " "	2.2
" 29	1,000 " " raw "	25 " " "	1.3
" 30	1,000 " " cooked "	25 " " "	Very faint.
" 31	1,000 " " raw "	25 " " "	Faint.

here represented, just as later authors⁵ have proved that a dose of sugar somehow prepares the normal organism so that a second dose is more perfectly assimilated.

Parenteral injection of pancreas extract may lower the sugar in blood or urine, like various other causes of intoxication or prostration, but no therapeutic benefit has ever been found from such treatment.⁶ The feeding of pancreas has never benefited diabetes, either in human patients⁷ or in dogs. Dogs are of value for these tests because they can eat much more in proportion to the body weight than human patients, but pancreas in either large or small quantity is found to produce glycosuria as readily as any other form of protein. Sandmeyer's observation that, when pancreatic juice is lacking, the improvement of digestion resulting from pancreas feeding may markedly increase glycosuria has been confirmed by Homans,⁸ and serves further to discredit the therapeutic usefulness of pancreas or pancreatic preparations given by mouth. Reach's claim that any raw meat may have the same glycosuric influence as pancreas, through some "toxic" action, appears confusing. The necessity of close personal supervision of feeding experiments by the investigator may again be mentioned. Some dogs have a strong repugnance for raw meat; others are ravenous for raw meat and will scarcely touch cooked meat. Fickleness of appetite and digestion is especially to be watched for in the Sandmeyer type of diabetes. The most plausible assumption to explain Reach's results is that his dogs either refused or vomited the cooked meat. The above experiments prove that there is no appreciable difference between cooked and raw meat in regard to either the glycosuria resulting directly from them or their influence on the assimilation of carbohydrate.

⁵ Hamman, L., and Hirschman, I. I., *Bull. Johns Hopkins Hosp.*, 1919, xxx, 306.

⁶ Allen,³ pp. 813-819, 855-857. Kleiner, I. S., and Meltzer, S. J., *Proc. Nat. Acad. Sc.*, 1915, i, 338.

⁷ Allen, F. M., Stillman, E., and Fitz, R., Total dietary regulation in the treatment of diabetes, Monograph of The Rockefeller Institute for Medical Research, No. 11, New York, 1919, Chapter IV.

⁸ Homans, J., *J. Med. Research*, 1915, xxxiii, 1.

Several experiments failed to show any sign of the wide glycosuric differences between proteins alleged by some authors in connection with the oatmeal "cure." There might be some possible interest in exact determinations of blood and urine sugar following the feeding of proteins differing in their content of sugar-forming and non-sugar-forming amino-acids, but the difficulties and uncertainties mentioned with regard to carbohydrate tests are still greater here. The above orientation experiments sufficed to exclude any differences of therapeutic importance.

Results of Immediate Protein Excess.

Dog B2-56.—Female; mongrel; white with brown patch over left eye; age 5 years; good condition; weight 16.5 kilos. Apr. 29, 1914. Removal of pancreatic tissue weighing 24.1 gm. Remnant about main duct estimated at 4.2 gm. (†). There was glycosuria for 3 days without feeding, probably because dissection about the pancreas remnant was followed by inflammation in it. Subsequently there was heavy continuous glycosuria on meat diet. May 14. Two tiny bits of tissue, weighing together only a small fraction of a gram, were removed from the pancreas remnant for examination. The remnant at this time did not appear on gross inspection as inflamed. Fasting was then imposed, May 14 to 23, but glycosuria was not reduced below 1.3 per cent and the diabetes was obviously uncontrollable. Meat was then given *ad libitum*, with resulting increase followed by decline of glycosuria and further loss of strength. May 28. The dog weighed 9.7 kilos, was too weak to stand, and was killed for autopsy.

Autopsy.—The pancreas remnant weighed 5.4 gm. and seemed normal in appearance and consistency. The gross autopsy was otherwise negative except for a large abscess in the right axilla, probably derived from a perforating ulcer at the elbow.

Microscopic Examination.—In the tissue removed on May 14 inflammation was limited almost entirely to broad bands of edematous fibrous tissue between lobules, infiltrated with leucocytes and also frequently hemorrhagic, while inside the lobules themselves there was little disturbance. The acini were normal and the islands markedly vacuolated. At autopsy the viscera, including the pancreas, seemed practically normal microscopically. Only slight thickening of trabeculae remained from the previous infiltration. Acini were normal; islands more extensively vacuolated than before and reduced in size and number.

The diabetes was evidently made more severe by inflammation, but even under these circumstances glycosuria was reduced to traces and was on the point of disappearing with 3 days of fasting following

the first operation. By care in diet at this time control of the diabetes could presumably have been achieved, and after subsidence of the inflammation the recovery of a high tolerance could have been expected. Excessive protein diet removed this possibility and, in conjunction with the inflammation, caused a much more rapid course of diabetes and cachexia than usual.

Results of Prolonged Protein or Protein-Fat Diets.

Examples were previously given⁹ of the existence of an apparent limit of protein tolerance in animals with the proper degree of diabetes; and when such a limit is exceeded, downward progress is natural and inevitable. As part of the protein molecule still remains available for nutrition, the decline of weight and strength is generally slower than on high carbohydrate diets, and life generally continues for several months, as illustrated in numerous former experiments. The above record of Dog B2-56 illustrates downward progress on protein as rapid as is usually seen on carbohydrate-rich diets, and also the facts that differences in the course of experimental diabetes are as marked as in clinical diabetes, and that the characteristic changes in the islands are not dependent on preformed carbohydrate in the diet. A less simple problem of protein feeding is presented under the following conditions.

The question arises whether, when an animal has diabetes, as demonstrated by glycosuria on carbohydrate feeding, but is able to eat protein to satiety without glycosuria, or when the protein ration is kept below the apparent tolerance and the full caloric requirements are supplied by addition of fat, diabetes is permanently avoided or merely delayed. It is essential that the test animals should have no changes in the pancreas tending to lower tolerance, and also that the actual permanency of the latent diabetes should be established and a tendency to spontaneous recovery excluded. With these precautions, it may be assumed that animals with simple resection of a portion of the pancreas are free from constitutional or other inherently progressive processes such as may be imagined in human patients. Tests with prolonged protein feeding in them are of the

⁹ Allen,³ p. 588, Dog 38, p. 777, Dog 154.

highest importance, to decide whether (notwithstanding the high content of potential carbohydrate in protein) the difference between protein and carbohydrate diet is absolute, or whether it is a difference of the kind above mentioned between sugar and starch, in that glycosuria is brought on merely more slowly but just as surely. Even with strict quantitative limitation of protein, the question may be stated on a broader basis with regard to the possibility of an impairment affecting the total metabolism in diabetes; namely, whether a diabetic organism can live out its full normal term of life at a full normal level of weight and metabolism by simple limitation of carbohydrate (preformed or from protein) in the diet, or whether the burden of general metabolism will suffice to wear out the weakened function so that an ultimate outbreak of frank diabetic symptoms will result. Such tests upon suitably chosen animals will contribute much toward the question of spontaneous downward progress in human diabetes and the efficacy of the classical treatment based on the idea of restriction of carbohydrate alone. Dogs are specially suited for such experiments, because of their relative insusceptibility to acidosis and other disturbances on pure protein or protein-fat diets.

The first dogs received upon beginning the investigation were set apart for these prolonged experiments. It was inevitable that deaths from distemper, rabies, and other accidents should spoil years of work in some cases, but by starting with a sufficient series of animals and substituting others as needed, some instructive long observations were obtained, of which the following four are the best examples.

*Dog B2-00.*¹⁰—Female; mongrel with some bull-terrier blood; brindle; age 3 years; good condition; weight 14 kilos. This dog was received on Oct. 25, 1913, and was subjected to five operations for removal of successive fractions of the pancreas, with periods of many months between so as to allow for any possible compensation by hypertrophy or alteration of assimilation from any cause, and with repeated tests of the carbohydrate tolerance at all stages. Tolerance was maintained for bread and soup with as much as 200 gm. of glucose up to Dec. 16, 1916, when the removal of only 0.1 gm. of pancreatic tissue brought on diabetes, so that bread and soup feeding alone sufficed for slight glycosuria. The dog

¹⁰ See photograph at end of Paper 5 (*J. Exp. Med.*, 1920, xxxi, 587).

passed through two pregnancies in quick succession, the second one terminating on July 16, 1917. The observations up to this point will be described in detail later. Throughout this time she was given 1 kilo of beef lung daily, but was not required to eat all of it; glycosuria was absent except in occasional tolerance tests.

The freedom from glycosuria continuing, the following plasma sugar tests were performed.¹¹ The time of feeding was between 9 and 10 a.m. daily. July 26. Body weight 13.2 kilos. Dog unwell. Plasma sugar before feeding 0.055 per cent; 2 p.m., 0.099 per cent; 5 p.m., 0.122 per cent; 8.30 p.m., 0.081 per cent. Nov. 27. Weight 14 kilos. 2 p.m. Plasma sugar 0.123 per cent. Dec. 3. Weight 14.5 kilos. Plasma sugar before feeding 0.067 per cent; 5 p.m., 0.108 per cent. Dec. 27. Weight 12.2 kilos. Plasma sugar before feeding 0.128 per cent; 2 p.m., 0.151 per cent; 5 p.m., 0.128 per cent.

TABLE III.
Dog B2-00.

Time.	Aug. 9, 1917.		Oct. 5, 1917.		Nov. 22, 1917.		Dec. 18, 1917.	
	Plasma sugar.	Urine sugar.	Plasma sugar.	Urine sugar.	Plasma sugar.	Urine sugar.	Plasma sugar.	Urine sugar.
	<i>per cent</i>	<i>gm.</i>	<i>per cent</i>	<i>gm.</i>	<i>per cent</i>	<i>gm.</i>	<i>per cent</i>	<i>gm.</i>
Before feeding. . . .	0.081	0	0.109	0	0.109	0	0.141	0
2 hrs. after " . . .	0.164	Very faint.	0.159	0	0.204	Faint.	0.400	3.55
4 " " " . . .	0.109	Faint.	0.145	Faint.	0.238	0.14	0.384	2.10
6 " " " . . .	0.135	"	0.152	0.14	0.125	0.11	0.357	3.95

Four tolerance tests also indicated a decline of tolerance during the above period of high protein diet. These tests consisted in giving, on the 4 days mentioned, an identical test diet of 200 gm. of bread, 150 gm. of glucose, and 100 gm. of beef lung, and determining the blood and urine sugars at the intervals shown in Table III.

Jan. 2, 1918. Diet changed to 400 gm. of lung and 50 gm. of suet. Jan. 15. Weight 12 kilos. Plasma sugar before feeding 0.139 per cent; 5 p.m., 0.161 per cent. Jan. 17. Diet changed to bones only. Jan. 21, 2 p.m. Plasma sugar 0.164 per cent. Jan. 28. Weight 11.1 kilos. Plasma sugar before feeding 0.133 per cent. Diet changed to 100 gm. of lung and 100 gm. of suet. Feb. 6, 2 p.m. Plasma sugar 0.155 per cent. Feb. 11. Plasma sugar before feeding 0.112 per cent; 2 p.m., 0.123 per cent. Feb. 14. Diet increased to 300 gm. of lung and 100 gm. of suet. Mar. 14. Diet increased to 500 gm. of lung and 100 gm. of suet.

¹¹ The methods of Lewis and Benedict (Lewis, R. C., and Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 61), and of Benedict (Benedict, S. R., *J. Biol. Chem.*, 1918, xxxiv, 203) have been used for all blood sugar analyses.

On protein diet from July 26, 1917, to January 15, 1918, the dog showed downward progress as evidenced by the increasing hyperglycemia and the tolerance tests. To exclude the possibility that such a hyperglycemic tendency might be due to something inherent in the dog, undernutrition was instituted on January 17 with a diet of nothing but fresh bones, and thereafter the quantity of protein was restricted, the maximum of 500 gm. of lung being attained by March 14. Hyperglycemia which is slow in onset, due to prolonged excess in protein or fat, is also slow in subsiding; but with the decline in body weight the blood sugar came gradually to the normal level. The last mentioned diet and the freedom from glycosuria continued to August, 1918.

*Dog B2-01.*¹⁰—Female; bull and fox-terrier mongrel; white with brown markings; age 2 years; good condition; weight 14 kilos. Received Oct. 25, 1913, and used at first like Dog B2-00 for removal of successive portions of pancreas.¹² The removal of 0.8 gm. of tissue in the final operation on Aug. 31, 1916, made the animal potentially diabetic.¹³ The tolerance was at first so high that bread diet with 300 gm. of glucose caused no glycosuria. It declined till glycosuria resulted from bread alone, then fluctuated according to the body weight, but on the whole fell in consequence of prolonged slight overfeeding, so that by June 30, 1917, there was hyperglycemia when the dog was fat on a diet of 400 gm. of lung and 200 gm. of suet. The weight was kept low and the tolerance high during the remainder of that year. In 1918 the dog was allowed to gain weight, on a diet which after Feb. 13 was 400 gm. of lung and 100 gm. of suet. The plasma sugar was normal (0.089 per cent) before feeding in the last analysis on Feb. 4.

*Dog D4-52.*¹⁰—Female; mongrel; yellow and white; age 3 years; good condition; weight 12 kilos. Nov. 24, 1916. Received with pups, which she reared. June 27, 1917. Removal of pancreatic tissue weighing 22.9 gm.; remnant about main duct estimated at 3.4 gm. ($\frac{1}{2}$). Glycosuria at first was heavy on feeding of bread and soup with glucose up to 200 gm., but soon ceased. July 12. 0.35 gm. of additional pancreatic tissue was removed, and the same experience with feeding was repeated. July 20. 0.2 gm. of additional pancreatic tissue was removed, and again the glycosuria from bread and 200 gm. of glucose was transitory. Aug. 3. A final fragment of 0.3 gm. of pancreatic tissue was removed. Glycosuria was absent on bread diet until Aug. 8, when the addition of 200 gm. of glucose made it heavy. On this glucose mixture the sugar excretion gradually diminished and ceased on Aug. 13.

¹² These experiments will be described later.

¹³ The subsequent feeding tests will be described later.

After Aug. 21, glucose was omitted and the diet was plain bread and soup. Slight glycosuria appeared on Oct. 10, and the diet was therefore changed to 500 gm. of beef lung and 100 gm. of suet. The same diet was continued regularly, though frequently the dog left considerable of it uneaten. By June, 1918, the weight had risen to a maximum of 12.6 kilos. Tests were performed at intervals (Table IV).

TABLE IV.

Dog D4-52.

Feeding of 500 gm. of lung, 100 gm. of suet, and 100 gm. of bread.

Date.	Weight.	Plasma sugar.			Urine volume.	Glycosuria.
		Before feeding.	3 hrs. after feeding.	6 hrs. after feeding.		
1917	kg.	per cent	per cent	per cent	cc.	per cent
Mar. 1	11.7	0.110	0.122	0.109	420	0
Oct. 25	11.6				580	2.44
Nov. 14	11.7	0.092	0.294	0.246	410	1.1

TABLE V.

Dog D4-52.

Feeding of 300 gm. of bread and 200 gm. of glucose.

Date.	Weight.	Plasma sugar.				Glycosuria (24 hrs.).
		Before feeding.	2½ hrs. after feeding.	4 hrs. after feeding.	6½ hrs. after feeding.	
1917	kg.	per cent	per cent	per cent	per cent	gm.
Aug. 8	11.5	0.149		0.312	0.092	6.75
" 15	11.6	0.101		0.172	0.161	0
Oct. 5	11.5	0.208	0.334	0.384	0.416	18.0

The tests mentioned above merely compare the diabetic condition on October 25 and November 14 with the normal state on the preceding March 1. As far as can be judged from glycosuria, there was gain rather than loss of tolerance from October 25 to November 14, though such differences may be accidental.

From the tests in Table V, it is seen that on August 8 (5 days after the last pancreas operation) the above glucose mixture caused hyperglycemia and glycosuria, which were brief, both being ended within the 6½ hour experimental period. By August 15, the bread and glucose

diet having been continued in the interval, tolerance had apparently been gained, so that glycosuria no longer resulted; but the curve of hyperglycemia, though lower, was longer. Delayed absorption is one possible factor here. The dog tired of glucose before diabetes was produced, so that after August it had to be discontinued, as already stated. On plain bread diet thereafter glycosuria was absent, but hyperglycemia was evidently present and tolerance was lost markedly. This was shown in the tolerance test of October 5, in which the blood sugar was high at the outset and ran a prolonged high course. Of the 18 gm. output, only 7.9 gm. were excreted during the $6\frac{1}{2}$ hour period, and the rest over night. A fast day was given on October 6, to allow recovery from the test. Nevertheless, glycosuria on plain bread and soup feeding began on October 10 as stated. This experiment is another illustration of downward progress on starch diet, and also of downward progress with hyperglycemia without glycosuria. A recuperative effort on the part of the pancreas remnant is manifest, but it was overcome by the excessive feeding.

Intravenous Glucose Tests.

The intravenous injections were given discontinuously, in a manner described in detail in a later paper. The dosage was constant, on the assumed normal weight of 12 kilos, without regard to the changes in actual weight. This meant the injection of 30 cc. of 10 per cent glucose solution every 15 minutes, in order to give 1 gm. per kilo per hour (Table VI). Feeding was omitted on each injection day and the day following. Excitement and other known causes of disturbance were avoided.

The experiment on August 6, 3 days after the last pancreas operation, seems to show the same characteristics as the above described feeding test on August 8; namely, beginning with an existing hyperglycemia, the blood and urine sugars rose quickly to high levels, but tended to fall toward the close, the relatively low percentages of the 7th and 8th hours being particularly striking.

The observations of November 19 and February 19 show the existence of hyperglycemia on prolonged carbohydrate-free diet, and downward progress of the diabetes notwithstanding absence of glycosuria.

TABLE VI.

Dog D4-52.

Intravenous glucose injections, 1 gm. per kilo per hour in 10 per cent solution (four injections per hour).

Time.	Plasma sugar.	Urine.	
		Volume.	Glucose.

Aug. 6, 1917. Weight 10.75 kilos.			
	<i>per cent</i>	<i>cc.</i>	<i>per cent</i>
Before injection.....	0.218		0
At end of 1st hr.....	0.500	16	2.64
" " " 2nd ".....	0.715	16	5.08
" " " 3rd ".....	0.475	54	2.84
" " " 4th ".....	0.590	52	1.54
" " " 5th ".....	0.415	76	0.74
" " " 6th ".....	0.270	78	1.81
" " " 7th ".....	0.202	94	1.45
" " " 8th ".....	0.125	82	0.82
1 hr. after injection.....	0.128	53	0.19
2 hrs. " ".....	0.130	12	Faint.
3 " " ".....	0.133	29	0
Glucose excreted.....			7.8 gm.

Nov. 19, 1917. Weight 11.7 kilos.			
Before injection.....	0.169		0
At end of 1st hr.....	0.555	36	1.93
" " " 2nd ".....	0.555	68	3.08
" " " 3rd ".....	0.435	82	1.49
" " " 4th ".....	0.370	132	0.79
" " " 5th ".....	0.370	98	0.39
" " " 6th ".....	0.356	126	0.28
" " " 7th ".....	0.370	127	0.55
" " " 8th ".....	0.370	105	0.39
" " " 9th ".....	0.384	105	0.36
" " " 10th ".....	0.322	101	0.42
1 hr. after injection.....	0.156	45	Faint.
2 hrs. " ".....	0.147	10	0
Glucose excreted.....			7.7 gm.

Feb. 19, 1918. Weight 12 kilos.			
Before injection.....	0.145		0
At end of 1st hr.....	0.417	20	4.77
" " " 2nd ".....	0.476	55	5.13
" " " 3rd ".....	0.500	114	3.39
" " " 4th ".....	0.525	109	3.45
" " " 5th ".....	0.500	102	3.40
" " " 6th ".....	0.475	140	2.78
" " " 7th ".....	0.455	90	4.35
" " " 8th ".....	0.384	87	3.23
1 hr. after injection.....	0.294	43	0.74
2 hrs. " ".....	0.170	19	Very faint.
Glucose excreted.....			25.7 gm.

Dog D4-69.—Male; black collie mongrel; age 3 years; good condition; weight 15.5 kilos. Jan. 5, 1917. Received. The removal of a kidney on this date was presumably without effect on the production of diabetes. Successive portions of pancreatic tissue were removed on Jan. 23, Feb. 23, Mar. 28, Apr. 19, May 8, June 1, June 27, and July 20. Tests of the tolerance were made between all the above operations, and diets of bread and soup with as high as 400 gm. of glucose, though causing more and more glycosuria, failed to maintain it permanently. After the last operation, which involved the removal of only 0.5 gm. of tissue, glycosuria was absent on bread and soup diet, but heavy at first with the addition of 100 gm. of glucose. As it tended to diminish, the glucose was increased on Aug. 8 to 200 gm., but again the heavy glycosuria diminished, became intermittent, and ceased. No glycosuria occurred after Aug. 15. Aug. 21. Glucose

TABLE VII.

Dog D4-69.

Feeding of bread and soup and 200 gm. of glucose.

Date.	Plasma sugar.			Glycosuria (24 hrs.).
	Before feeding.	3½ hrs. after feeding.	5½ hrs. after feeding.	
1917	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>gm.</i>
Aug. 8	0.133	0.344	0.310	4.5
" 9	0.116	0.238	0.264	Trace.
" 15	0.110	0.217	0.212	0
Oct. 5	0.200	0.525	0.435	24.8

was discontinued, and the diet thereafter was plain bread and soup. The weight had risen by Oct. 5 to 17.6 kilos. Tolerance tests were performed during this period (Table VII).

Though 2 fast days were imposed following Oct. 5, the former sugar freedom on bread diet was no longer possible, the glycosuria in repeated attempts being persistent and increasing. Therefore, beginning Oct. 17, the diet was 100 gm. of suet and quantities of lung varying from 400 to 1,000 gm. Oct. 25 to 30. A diet of 500 gm. of lung and 100 to 150 gm. of bread caused continuous glycosuria. After this the diet was 500 gm. of lung and 100 gm. of suet, with continuous absence of glycosuria except on a few test days.

Nov. 14. Addition of 100 gm. of bread to the diet caused glycosuria of 0.3 per cent in 350 cc. of 24 hour urine. The plasma sugar before feeding was 0.127 per cent; 3 hours after feeding, 0.244 per cent; 6 hours after feeding, 0.138 per cent.

Intravenous tolerance tests were performed as shown in Table VIII.

Experiments like those on Dogs B2-00, B2-01, D4-52, and D4-69, with their requirements of prolonged care in diets, daily urinalyses, and attention to numerous details, are full of difficulties. These four dogs were the survivors of a series in which the attempt was made to reproduce the conditions of human patients as closely as possible. The above records extend to the summer of 1918, when the writer entered military service, and the dogs were left on the

TABLE VIII.

Dog D4-69.

Intravenous injection of 1.3 gm. per kilo per hour of a 10 per cent solution of glucose.

Time.	Aug. 6, 1917. (Weight 16.2 kilos.)			Dec. 4, 1917. (Weight 14.2 kilos.)			Feb. 19, 1918. (Weight 15.5 kilos.)		
	Plasma sugar.	Urine.		Plasma sugar.	Urine.		Plasma sugar.	Urine.	
		Volume.	Glucose.		Volume.	Glucose.		Volume.	Glucose.
	per cent	cc.	per cent	per cent	cc.	per cent	per cent	cc.	per cent
Before injection.	0.106	10	0	0.104		0	0.154	32	0
After 1st hr.	0.278	20	1.56	0.715	100	2.84	0.625	46	4.83
“ 2nd “	0.294	30	2.87	0.990	144	4.45	0.800	214	4.00
“ 3rd “	0.312	64	1.71	0.990	222	3.65	1.000	252	4.35
“ 4th “	0.270	106	0.76	1.000	300	2.80	1.180	274	3.70
“ 5th “	0.218	110	0.42	0.844	220	2.93	1.110	281	4.25
“ 6th “	0.204	76	0.53	0.844	265	2.43	1.050	270	4.55
“ 7th “	0.183	128	0.51	0.625	222	2.71	1.000	222	4.55
“ 8th “				0.715	216	3.82	0.950	278	5.13
1 hr. after last injection.	0.082	90	Trace.	0.370	60	3.60	0.525	78	5.88
2 hrs. “ “ “	0.098	82	0	0.217	66	0.49	0.322	10	5.13
Glucose excreted.....			4.5 gm.			55.2 gm.			80.4 gm.

Both the feeding and the intravenous tests indicated gradual loss of tolerance in the absence of glycosuria, first on starch and later on protein-fat diet. The sugar curves of Feb. 19, 1918, were not only the highest and most prolonged, but also with the initial figure of 0.154 per cent indicated that hyperglycemia was now constant on the regular diet of lung and suet. In the final blood taken at the height of digestion at 2.30 p.m. on Mar. 20, 1918, the plasma sugar was 0.256 per cent, still without glycosuria. The weight was gradually falling, and was 14.75 kilos at this time.

diets stated under the care of the animal attendants. They were seen once during the winter, sufficiently to learn that all four were free from glycosuria; three of them were vigorous and fat, but Dog D4-69 was thin and in rather poor condition. In the late summer of 1919, two of the dogs were found to have hyperglycemia and glycosuria, and were therefore transferred from The Rockefeller Institute to the writer's clinic. The two which were sugar-free were cared for at the farm of the Institute in New Jersey.

The animal in worst condition was Dog D4-69, which was thin, with hopeless hyperglycemia and glycosuria, and which died of diabetes on October 10, 1919. Dog B2-01 was strong and fat at a weight of 14.8 kilos, but showed heavy sugar and acetone reactions. Glycosuria was at first abolished by fasting, but returned, owing to persistence of hyperglycemia when attempts were made to feed. Rigorous undernutrition at this time might have proved successful, but the mistaken laxness, due to the deceptive strength and fatness of the animal, soon ended in a hopeless condition, which was uncontrollable by fasting, so that death occurred November 15, 1919. The record of the terminal period is given in the next paper. The other two dogs were in excellent condition when visited at the farm on November 3, 1919. Plasma sugar analyses on that day were as follows: Dog B2-00, before feeding, 0.092 per cent; 4 hours after feeding, 0.136 per cent; Dog D4-52, before feeding, 0.093 per cent; 4 hours after feeding, 0.123 per cent.

It will be seen that the experiment consisted in taking four dogs with different grades of diabetes, which had been produced by sufficiently long experimental procedures to rule out accidental influences as far as possible, and had been tested in various feeding and injection experiments, and then placing them on nearly identical diets on which they were free from glycosuria for extended periods. The ultimate outcome could in the main have been predicted from the preliminary observations.

Dog D4-69 had continuous hyperglycemia in the spring of 1918, and in the absence of extraneous interfering factors such a condition in dogs always leads to manifest and fatal diabetes. Loss of weight may have been due to the latent diabetes or to indigestion or other causes; it evidently postponed the outbreak of active dia-

betes longer than usual. Dog B2-01 exhibited hyperglycemia and lowered tolerance when obese in the summer of 1917, and it was therefore to be expected that a similar result would follow when she became fat on the same protein ration in 1918-19. These records illustrate that in dogs, as in human patients, a true recuperative power is limited to the earlier stages of diabetes, and when diabetes has lasted many months or years the assimilation may undergo apparent fluctuations according to the diet and weight but is not capable of any great restoration in an absolute sense. The final purpose was, after the expected onset of diabetes, to check this as before by fasting and reduced diet, and then prove that the animals could be kept indefinitely in the undernourished condition free from diabetes or downward progress, on the same protein ration; in other words, to show the influence of dietary fat and body weight in causing downward progress. This fact was established by other experiments,¹² but the opportunity of testing the permanency of such arrest of diabetes and the feasibility of protecting the assimilation by undernutrition extending over many years or the full lifetime of the dogs was lost.

Dogs B2-00 and D4-52 had milder diabetes. Both showed downward progress on excessive diets of carbohydrate or protein, and both illustrated the fact, often observed in human patients, that when mild diabetes is checked by a diet within the actual tolerance, hyperglycemia may persist for a long time but gradually subsides. The question was what would happen to such animals if they were allowed to live indefinitely on a diet which seemed to be within the tolerance. The result could not be predicted in advance, and the outcome to date establishes the following conclusions.

1. No inherent downward progress is perceptible. Dog B2-00 has been kept for 6 years, and has been demonstrably diabetic for 3 years. The downward progress observed in other animals, and also in these animals on excessive diets, is purely the result of food injury; in other words, to functional overstimulation of the pancreas as an endocrine organ.

2. The benefit of the classical treatment of diabetes is confirmed. With the susceptibility of both these animals to injury from excess of either carbohydrate or protein demonstrated, it is evident that

this injury was checked when carbohydrate was omitted, protein restricted, and a full caloric diet made up by the use of fat. Fat is evidently less injurious than carbohydrate or protein, and its harmfulness is chiefly noticeable in the more severe grades of diabetes. It is safer for these animals to be obese on a fat diet than to eat carbohydrate or a carbohydrate-forming food such as protein. By inference, fat is not a direct source of carbohydrate.

3. The future outcome in these two animals holds several possibilities. (a) One question concerns the degree and permanence of their assimilative power. Other experiments justify the assumption that by undernutrition their tolerance could be greatly raised, so that they might take considerable protein and carbohydrate, and by increased obesity the tolerance could be further lowered, probably to the point of glycosuria on their present protein ration. The question is whether their tolerance at their present weight is high enough for permanent assimilation of the limited carbohydrate derivable from their present diet; whether the difference between fat and protein is absolute or merely a matter of time, like the differences between glucose and starch or between starch and protein; and therefore whether diabetes is primarily a deficiency of the total metabolism or of carbohydrate metabolism alone. (b) The plasma sugars under present conditions are not absolutely normal. Particularly in Dog B2-00 the figure of 0.136 per cent during digestion of protein is above normal, but yet is within the limits permitted in numerous diabetic patients. There is a question whether this slight and temporary overload of each day can be borne indefinitely by the pancreas, or whether it is the first small sign of a breaking strain. (c) In addition to the simple prolonged functional wear and tear, other influences such as age enter in, and it is of interest to know whether senility will bring an onset of diabetes, as it seems to do in so many human patients. If necessary, the direction of progress in the animals can be judged at any time by tolerance tests, which were instituted in the first place in order to permit of such comparisons, but it seems better to avoid this source of possible injury. As these dogs bear such a close resemblance to mildly diabetic patients kept sugar-free by restriction only of sources of carbohydrate, it seems most valuable to learn whether they can remain fat and lazy indefinitely

with impunity, or whether even the mildest diabetes will ultimately undergo aggravation from luxus diets.¹⁴

CONCLUSIONS.

No specific differences were observed between the glycosuric effects of different kinds of protein. Other incidental observations were noted in connection with the records of individual animals. General conclusions are deferred to the close of the series.

¹⁴ Both these dogs recently died and were autopsied. Dog D4-52 had a large calculus in the bladder but otherwise appeared to be in excellent condition. No cause of death was found. Dog B2-00 was more closely observed; there was a history of refusal of food, and drinking and vomiting of water. The urine in the bladder was heavy with sugar; the subcutaneous tissue and peritoneum contained abundant fat, and the liver was intensely fatty. It is therefore certain that the death of this animal was due to acidosis. The same possibility exists for Dog D4-52 but is unproved. The results indicate that luxus diets lead to a fatal termination in diabetes.

EXPERIMENTAL STUDIES ON DIABETES.

SERIES I. PRODUCTION AND CONTROL OF DIABETES IN THE DOG.

4. CONTROL OF EXPERIMENTAL DIABETES BY FASTING AND TOTAL DIETARY RESTRICTION.

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In a previous publication the writer reported the results of attempts to produce a satisfactory experimental reproduction of clinical diabetes and a study of its nature, which was made to obtain some clue leading to its therapeutic control.¹ This investigation was carried to a point where it was believed, as stated in the preface, that the possession of the necessary working model of the disease had made the cure of diabetes a feasible laboratory problem. Out of numerous lines of therapeutic approach suggested from the literature, speculation, or experiment, two seemed worth following but had to be left unexplored. One of these consisted in attempts to stimulate or strengthen the pancreatic function in some direct manner, and it is still hoped to proceed in this direction at some later date. For certain reasons it was desirable to follow the other path first.

The phenomenon which suggested this latter research was the prevention or cessation of diabetes with ligation of the pancreatic duct.² It was at first suspected that this was due to improvement of the internal pancreatic function, through stoppage of the external secretory activity or even through structural changes involving increase of island tissue. When the work was finished, there was leisure for the following comparison with other observations. In Dog 55³ diabetes ceased with the onset of distemper and the attendant loss of appetite and weight. In Dog 57, fasting for a week prior to operation seemed to prevent the onset of glycosuria. Likewise peritonitis and other infections often hindered the occurrence of glycosuria, though in some instances diabetes appeared as usual, notwithstanding the presence of peritonitis. Several other dogs⁴ showed absence or cessation of glycosuria in consequence of illness, fasting, and loss of weight.

¹ Allen, F. M., *Studies concerning glycosuria and diabetes*, Cambridge, 1913.

² Allen,¹ Chapter XXII.

³ Allen,¹ p. 493.

⁴ Allen,¹ pp. 771-772.

Also in an animal with fairly severe and advanced diabetes,⁵ glycosuria was twice stopped by fasting, and downward progress in consequence of the overfeeding during the interval was demonstrated by the lower tolerance for protein at the close of the second as compared with the first fast. There are reports in the literature of the apparent cessation of diabetes with an intercurrent cachectic disorder,⁶ and also of the benefit of the occasional therapeutic fast days introduced by Bouchardat, Cantani, and Naunyn. In addition, shortly before the beginning of the new research, Joslin emphasized the subsidence of diabetic symptoms in a severe case after the onset of tuberculosis with emaciation.⁷ It thus seemed possible that the cessation of glycosuria from ligation of the pancreatic duct resulted from impaired food absorption and undernutrition. Homans⁸ confirmed the phenomenon itself and demonstrated this as the true explanation by direct experiments.

The first opportunity was taken to try reduction of weight and total metabolism for the treatment of diabetes. The first dogs were chosen for the longest experiments, as explained in the preceding paper. (It so happened that Dog B2-05 furnished another illustration of the cessation of glycosuria with distemper.) The tests consisted in taking dogs, generally with much smaller pancreas remnants than those with which downward progress and death were found on full diets in the preceding paper, and determining to what extent active symptoms and impairment of assimilation could be prevented by fasting and total dietary restriction. Many examples are available and are mentioned incidentally in other papers, because the method was used as a routine for controlling diabetes. As the longest records are the most instructive for the present purpose, three experiments of 1 to $1\frac{3}{4}$ years in duration are here summarized as successful cases. Before proceeding to these, brief consideration may be given to the status of the so called "hunger glycosuria" in this connection.

Dog B2-52.—Female; bull-terrier mongrel; brindle; excellent condition; weight 12.8 kilos. Apr. 24, 1914. Removal of pancreatic tissue weighing 20.4 gm. Remnant about main duct estimated at 3.1 gm. (slightly over $\frac{1}{8}$). Glycosuria

⁵ Allen,¹ Dog 64, pp. 354–361, 480.

⁶ Allen,¹ p. 800 ff.

⁷ Benedict, F. G., and Joslin, E. P., *Carnegie Inst. Washington, Pub. No. 136*, 1910. Joslin, E. P., *Treatment of diabetes mellitus*, Philadelphia and New York, 2nd edition, 1917, 409.

⁸ Homans, J., *J. Med. Research*, 1915, xxxiii, 1.

was absent till bread and soup were fed on Apr. 30. It was maintained till May 7 by the addition of as much glucose as possible, but then ceased largely because of the dog's distaste for sugar. May 12 to 15. Fasting was imposed. The feeding of bread and soup with 50 to 75 gm. of glucose daily then caused glycosuria of 0.7 per cent on the 1st day, but none thereafter, and a second operation was necessary to make the dog diabetic.

This dog is representative of several tested in relation to hunger glycosuria, some of which were previously mentioned.⁹ The point involved is the seeming conflict between the use of fasting and under-nutrition for raising tolerance, and the experience of Hofmeister and others concerning the tendency to glycosuria created by starvation and malnutrition. When a dog on the border-line of diabetes refuses to eat enough to keep up glycosuria and break down tolerance, it might seem a promising plan to fast for a number of days and then feed bread and glucose, with the double idea that the glycosuric tendency would be increased by the fast, and also that the animal would eat more. Hofmeister's work is confirmed in these dogs much more strikingly than in normal dogs, and heavy glycosuria often occurs on feeding bread either alone or with sugar. The same phenomenon has frequently been witnessed in dogs changed suddenly from a protein or fat diet to a carbohydrate diet. But no matter how heavy the glycosuria, it is transitory just as in Hofmeister's normal dogs, and it has never been possible to produce diabetes thus in any animal which was non-diabetic on the same diet before the fast. The phenomenon seems to illustrate some state of unpreparedness of the body for the unaccustomed carbohydrate flood, but it does not represent any true diabetic tendency or any exception to the general rule that the pancreatic function is strengthened by fasting.

*Dog B2-25.*¹⁰—Male; mongrel; tall, rough haired, brown and white; age 3 years; moderately well nourished; weight 18.2 kilos. Dec. 16, 1913. Removal of pancreatic tissue weighing 30.1 gm. Remnant about main duct estimated at 2.6 gm. ($\frac{1}{12}$ — $\frac{1}{5}$). The further record is contained in Table I. Dec. 3, 1914. In excellent health and strength; accidental death.

Autopsy.—Negative grossly and microscopically. Pancreas remnant, fully normal in appearance and consistency, weighed 8.25 gm. Microscopically, acini normal; islands small and scarce, free from vacuolation.

⁹ Allen,¹ pp. 586–587. Cf. Paper 3, p. 557; also Dog B2-51, p. 578.

¹⁰ See photograph at end of Paper 5 (*J. Exp. Med.*, 1920, xxxi, 587).

The animal lived approximately a year, was most of the time hungry and excessively thin, but vigorous and lively, in contrast to the weakness of diabetes. Tolerance was very low at the outset, corresponding to the small pancreas remnant; but instead of downward progress there was a very great gain in assimilation. The clinical absence of diabetes was corroborated by the intact state of the islands.

TABLE I.

Dog B2-25.

Date.	Body weight.	Diet.	Urine.	
			Volume.	Glucose.
1913	kg.		cc.	per cent
Dec. 17	18	Fasting.	1,025	0
" 18		200 gm. of meat.	1,500	1.0
" 19		Fasting.	1,700	0.6
" 20		"	875	Faint.
" 21-26		"		0
To May, 1914	Down to 11.	From 30 gm. of lung and 30 gm. of suet to 120 gm. of lung and 120 gm. of suet.		Traces of glycosuria at first on the higher diets; later none.
" Dec., 1914	Increase to 17.	Pancreas or lung increasing as described in preceding paper,* up to 1,400 gm. daily.		Glycosuria absent till Nov. 25-27; then 4.1 to 8.2 gm.; stopped by a fast day.

* Allen, F. M., *J. Exp. Med.*, 1920, xxxi, 556.

Dog B2-51.—Male; mongrel; yellow and white; age 1½ years; moderately well nourished; weight 9.75 kilos. Mar. 31, 1914. Removal of pancreatic tissue weighing 20.1 gm. Remnant about main duct estimated at 2.8 gm.(½). Beginning Apr. 2, a diet of 200 cc. of milk daily caused glycosuria as high as 2.6 per cent, and on Apr. 5 the change to bread and soup diet resulted in glycosuria of 5 per cent; but this gradually subsided and could only be maintained by the addition of 50 gm. of glucose, which was as much as the dog would take on account of a decided distaste for sugar. With increasing indigestion and loss of weight, glycosuria ceased on Apr. 20.

Apr. 21. Body weight 8 kilos; opening the abdomen revealed obvious marked hypertrophy of the pancreas remnant, from which 1.5 gm. of tissue were removed. Glycosuria was then absent on meat diet. Apr. 26. An abrupt change to bread and soup produced glycosuria as usual for 1 day; it then remained absent even with the addition of 50 gm. of glucose on Apr. 30.

May 1. An additional 1.8 gm. of pancreatic tissue was removed, the body weight being 8.1 kilos. A slight glycosuria ensued on that day, then was absent on beef lung, and after May 7 on bread and soup with 50 gm. of glucose. The emaciated and cachectic condition was a sufficient explanation; when the full diet was forced it was probably largely lost through diarrhea, and on May 12 the weight was down to 6.4 kilos. Accordingly meat diet *ad libitum* was then resumed, with gradual

TABLE II.
Dog B2-51.

Date.	Body weight.	Diet.		Glyco- suria.
	kg.			per cent
1914				
July 1-9	8.7-7.6	100 gm. of lung.		0
" 10-22	7.6-7.0	200 " " "		0
" 23-30	6.9-6.75	250 " " "		0
" 31-Aug. 9	6.7-6.6	300 " " "		0
Aug. 10-14	6.6-6.5	325 " " "		0
" 15-19	6.5-6.7	100 " " "	100 gm. of suet.	0
" 20-26	6.8-6.5	100 " " "	100 " " "	0
" 27-Oct. 6	6.6-7.2	100 " " "	75 " " "	0
Oct. 7-15	7.2	100 " " "	50 " " "	0
" 16-Nov. 5	7.2-6.8	100 " " "	30 " " "	0
Nov. 6-Dec. 1	6.9-7.2	100 " " "	30 " " " 50 gm. of lard.	0
Dec. 2-13	7.2	150 " " "	30 " " "	0
" 14-21	7.2-6.6	150 " " "		0
" 22-Jan. 3,				
1915	6.5	150 " " "	50 " " lard.	0
1915				
Jan. 4-Feb. 17	6.6-8.0	150 " " "	100 " " "	0
Feb. 18-Mar. 1	8.0-8.1	200 " " "	50 " " "	0
Mar. 2-18	8.2-8.9	150 " " "	100 " " "	0
" 19	8.8	400 " " "		Trace.
" 20	8.8	400 " " "		0.16
" 21	8.6	Fasting.		0.36
" 22	8.4	100 gm. of lung.		0.25
" 23	8.4	100 " " "		0

gain in weight and health. By June 24, the weight had risen to 9.3 kilos. Glycosuria, which had remained absent before, suddenly appeared and continued for 5 days, the highest percentage being 1.2. It was stopped by 2 fast days; the diet and progress after these fast days are shown in Table II. Mar. 26, 1915. Accidental death.

Autopsy.—The pancreas remnant, normal in appearance and consistency, weighed 2.75 gm. Islands were rather few and small, but within normal limits.

Occasional cells in them showed slight vacuolation. Liver moderately fatty. Otherwise autopsy negative, including microscopic examination of thyroid, liver, kidneys, adrenals, and hypophysis.

Pancreatic tissue removed by operation Apr. 21, 1914, normal except for slight vacuolation in occasional island cells. Pancreatic tissue removed May 1, 1914, normal except for slight inflammatory reaction in places; no vacuolation in islands.

It is probable that the dog might have been made diabetic after the first operation if the sugar feeding could have been forced as in some other animals. This is suggested by the glycosuria and the anatomic evidence of overtaxed function in the island cells; but at the same time hypertrophy of the remnant and fall of body weight had the opposite influence, and repugnance to carbohydrate, indigestion, and diarrhea closed the attempt. Prior to the second operation, glycosuria was absent, and correspondingly the islands showed no vacuolation. Shortly preceding death there was slight glycosuria on protein diet, and this was paralleled as usual by slight hydropic changes in the islands. This experiment is one illustration that neither the size of the pancreas remnant, inflammatory changes, the length of time since a preceding operation, nor the character of the diet (carbohydrate or protein) govern this change in the islands, but only the presence or absence of active diabetes.

The record from May 1 to July 1, 1914, illustrates absence of diabetes in an animal in an emaciated condition on pure protein diet, and the loss of tolerance and outbreak of active symptoms when weight was gained on this diet.

The subsequent record shows the absence of diabetes on a restricted protein-fat diet, frequently adjusted in minor details to suit the dog's appetite and digestion, but so planned as to keep the body weight generally low. The animal was lively and fairly strong meanwhile. There was no tendency to real recovery, for when the weight was allowed to rise at the end, it was found that 400 gm. of lung sufficed to cause slight glycosuria. The slight vacuolation found in the islands resulted from such periods of overfeeding. The principal point of the experiment was the ability to keep a thin animal with diabetes of this degree of severity free from symptoms, without evidence of downward progress or indications that the condition could not have been continued indefinitely.

Dog D4-28.—Male; mongrel; yellow and white; age 2 years; good condition; weight 12 kilos. Sept. 28, 1916. Removal of pancreatic tissue weighing 25.3 gm. Remnant about main duct estimated at 2.3 gm. ($\frac{1}{2}$). As this dog had been born and raised on the Institute farm and was known to have lived all his life on bread and cereals, after operation advantage was taken of the opportunity to test the effects of fasting and fat diets, as will be described in a subsequent paper on acidosis. Neither glycosuria nor acidosis occurred, and the plasma sugar on Oct. 2 was 0.128 per cent, on Oct. 3, 0.092 per cent, and on Oct. 5, 0.101 per cent. After Oct. 5 the dog was kept on rather low protein-fat diets, maintaining a body weight of 10.5 to 10.2 kilos.

Oct. 29. A pan of bread and soup was fed, causing the excretion of 2.39 per cent sugar in 663 cc. of urine.

TABLE III.

Dog D4-28.

Date.	Urine.		Remarks.
	Total nitrogen.	Ammonia nitrogen.	
1917	gm.	gm.	
Jan. 28	2.14	0.08	
" 29	3.73	0.19	
" 30	1.34	0.11	
" 31	2.33	0.11	
Feb. 1	1.55	0.10	
" 2	2.98	0.30	Total nitrogen in the feces for the period 4.46 gm.
" 3	2.62	0.27	

Oct. 30. A diet was fed of 200 gm. of beef lung, 200 gm. of suet, and 75 gm. of bread, without glycosuria. The plasma sugar before feeding was 0.184 per cent. Taken at 2 hour intervals for 12 hours after feeding it was 0.184, 0.200, 0.222, 0.213, 0.184, and 0.172 per cent. The following morning before feeding it was 0.179 per cent.

Nov. 2. The diet was 200 gm. of lung, 150 gm. of lard, and 100 gm. of bread. The plasma sugar before feeding was 0.143 per cent. Taken at 2 hour intervals for 12 hours thereafter it was 0.166, 0.180, 0.200, 0.208, 0.182, and 0.182 per cent, without glycosuria.

Nov. 3, 10 a.m. Plasma sugar 0.148 per cent. The dog was then fed only 200 gm. of lung. 5 p.m. Plasma sugar 0.143 per cent.

Beginning Nov. 4, on a diet of 200 gm. of lung and 100 gm. of suet, the dog gradually gained weight up to 11.3 kilos on Dec. 4. On that day faint glycosuria appeared, and was absent the next day. On Dec. 6 there was excretion of 0.74 per cent glucose in 124 cc. of urine, on Dec. 7, 0.59 per cent in 136 cc. of urine, and

on Dec. 8, 1.81 percent in 396 cc. of urine, with a trace of acetone. With 1 day of fasting there were only traces of sugar and acetone, and a 2nd fast day cleared up both. Then the feeding of 100 gm. of suet failed to bring back either sugar or acetone. These remained absent thereafter on a diet of 100 gm. of lung and such

TABLE IV.

Dog D4-28.

Date.	Urine.		Remarks.
	Total nitrogen.	Ammonia nitrogen.	
1917	gm.	gm.	
Apr. 10	1.69	0.10	
" 11	1.88	0.27	
" 12	1.24	0.10	
" 13	2.40	0.19	
" 14	2.46	0.25	
" 15	1.60	0.16	
" 16	1.28	0.12	
" 17	3.32	0.28	
" 18	1.97	0.20	Total nitrogen in the feces for the period 1.69 gm.
" 19	2.08	0.22	

TABLE V.

Dog D4-28.

Date.	Urine. Total nitrogen.	Date.	Urine. Total nitrogen.
1917	gm.	1917	gm.
Oct. 5	1.12	Oct. 13	—
" 6	1.64	" 14	2.18
" 7	2.04	" 15	2.06
" 8	1.78	" 16	1.82
" 9	1.74	" 17	1.60
" 10	2.36	" 18	1.96
" 11	2.18	" 19	3.24
" 12	—	" 20	2.52

small quantities of suet as the dog chose to eat. Dec. 27. Plasma sugar 0.084 per cent, with hemoglobin (Fleischl-Miescher) 114 per cent. Jan. 18, 1917. Plasma sugar 0.083 per cent, with hemoglobin 111 per cent. The weight during this time up to Jan. 27 ranged from 10.4 to 11 kilos.

Jan. 27 to Feb. 3. A metabolism experiment was conducted on a diet of 100 gm. of lung and 100 gm. of suet. The dog was catheterized on these two dates,

but in the interim passed urine spontaneously and regularly. Feces were combined and analyzed in one lot for the entire period. The results are given in Table III.

Beginning Mar. 10, at a body weight of 10.4 kilos, the above diet was increased by 25 gm. of bread. After Mar. 13, this was changed to 50 gm. of lung, 50 gm. of bread, and 100 gm. of suet. Apr. 10 to 19. Urinalyses were performed on the latter diet (Table IV).

The body weight gradually rose from 10 kilos on Mar. 20 to 10.8 kilos on June 30. June 15. The plasma sugar before feeding was 0.081 per cent; 4 hours after feeding, 0.161 per cent. Suet was then omitted; this made the diet only 50 gm. of lung and 50 gm. of bread. The weight was thus reduced by July 17 to 9.75 kilos. After that, 50 gm. of suet were resumed, but were omitted at times as the dog tired of it, so that the body weight was kept between 9.2 and 10 kilos. Oct. 5 to 20. A series of urinalyses, omitting feces, was performed on the usual diet of 50 gm. of lung, 50 gm. of bread, and 50 gm. of suet (Table V).

Nov. 26. The above diet was fed about noon. 3 p.m. Plasma sugar 0.118 per cent. Beginning Nov. 27, bread alone was fed, in increasing quantities, from 150 gm. on the 1st day to 300 gm. on Nov. 30, without glycosuria.

Dec. 1, 12.30 p.m. The dog was fed 300 gm. of bread with 100 gm. of glucose, without glycosuria. The plasma sugar before feeding was 0.13 per cent, and at 4 p.m., 0.216 per cent. Dec. 2. Diet the same. Dec. 3. The glucose was increased to 150 gm., still without glycosuria. Dec. 4. Plain bread and soup mixture was fed. Dec. 5 to 7. 200 gm. of glucose were added daily, with glycosuria never above 0.61 per cent. Dec. 8. The plasma sugar was 0.161 per cent, and the diet was changed to lung *ad libitum*. The body weight at this time was 9 kilos.

Beginning Dec. 14, 100 gm. of suet were added, and on 2 days, *viz.* Dec. 14 and 26, a pan of bread and soup was allowed in addition, without glycosuria. The weight thus rose to 10.8 kilos.

A test with carbohydrate was then performed, in comparison with the one a month previous at a lower weight. Glycosuria was absent on bread diet. On Jan. 3, 1918, when 300 gm. of bread with 100 gm. of glucose were fed to compare with Dec. 1, the plasma sugar before feeding was 0.154 per cent and at 4 p.m., 0.452 per cent, with excretion of 3.8 per cent sugar in 700 cc. of urine for the 24 hours.

Jan. 4. Glycosuria ceased on a diet of 300 gm. of lung and 100 gm. of suet. Jan. 5. On feeding plain bread and soup there was excretion of 3.45 per cent sugar in 415 cc. of urine.

Thereafter the diet of 50 gm. of lung, 50 gm. of suet, and 50 gm. of bread was resumed. A gradual fall in weight ensued, to 9.75 kilos on Jan. 28, 8.75 kilos on Feb. 21, 8.3 kilos on Mar. 11, and 7.4 kilos on June 11. Glycosuria was constantly absent, and up to June 8 the dog maintained the utmost activity. The urine, as collected under the cage, then became increasingly bloody. The cause

was supposed to be bloody diarrhea, due to deficient diet, as described by Rosenheim. The diet was therefore enriched with an abundance of fresh meat, bones, milk, eggs, and yeast, to supply any missing elements. Nevertheless, the condition became worse. June 12. The animal was found dying and was killed for autopsy. The plasma sugar of the heart's blood was 0.159 per cent, the carbon dioxide capacity 32.8 volumes per cent, without acetone or other evidence of acidosis.

Autopsy.—The cause of death was found to be urinary obstruction from a large calculus impacted in the urethra near the bladder, with several smaller stones lodged behind it. More or less urine had been passing up to the day of death, but the bladder and both ureters and kidneys were distended with bloody urine, which had also infiltrated extensively about the neck of the bladder. The intestine was normal throughout and there was no blood in its contents anywhere. The other viscera were negative. The pancreas remnant, normal in appearance and consistency, weighed 3.5 gm.

Microscopic Examination.—The kidneys were inflamed and infiltrated with mononuclear cells chiefly in the cortex and polymorphonuclears chiefly in the medulla. The Armani or Ehrlich vacuolation was also evident in some tubules. The liver was normal and its cells contained no visible fat. The pancreas remnant was entirely normal and free from fibrosis. The acini were regular and well filled with zymogen, and the islands normal in number and size and free from vacuolation. The other organs were normal.

As the experiment was a long one, the opportunity was taken to use it for several purposes simultaneously. Aside from the acidosis observations mentioned, the following points were included. As low protein diets, sometimes in conjunction with undernutrition, are an occasional feature of diabetic treatment, it was desirable to test the feasibility of such a program in the dog. This was especially desirable in regard to any possible doubts still persisting from the work of Munk,¹¹ Rosenheim,¹² and Zuntz and Magnus-Levy,¹³ though it seems generally accepted that the difficulties reported by these investigators were due not to protein deficiency but to other unsuitable features in the diets used. One of these features is considered to be the use of meat powder in Munk's 10 week experiment. Rosenheim noticed decline of appetite and digestion within 8 weeks, perhaps

¹¹ Munk, I., *Arch. Physiol.*, 1891, 338; abstracted in *Jahresb. Fortschr. Thier-Chem.*, 1892, xxi, 365.

¹² Rosenheim, T., *Arch. Physiol.*, 1891, 341; abstracted in *Jahresb. Fortschr. Thier-Chem.*, 1892, xxi, 365.

¹³ Zuntz, N., and Magnus-Levy, A., *Arch. ges. Physiol.*, 1891, xlix, 438.

due to the rice used, and then obtained a fatal result by changing to an excessive fat ration. The dog described above (No. D4-28) had presumably some tendency to impaired digestion created by the removal of $1\frac{1}{2}$ of the pancreas, yet suffered none of the disturbances described by these authors. A little bone-meal daily prevented diarrhea and presumably contributed some mineral salts. From March 14 to November 27, 1917, the dog remained in excellent health and spirits on 50 gm. of beef lung (cooked), 50 gm. of bread, and 50 to 100 gm. of raw beef suet. Weight was gained whenever the calories were sufficient and was lost in normal manner whenever the dog tired of too much suet. After January 6, 1918, there was a gradual and regular loss of weight up to June, on the undernutrition diet of 50 gm. of lung, 50 gm. of bread, and 50 gm. of suet. During these long periods there was no sign of any specific disorder due to the reduction of urinary nitrogen to approximately 2 gm. daily.

During this same time, the animal served as a control for other dogs on high fat diets, as will be described in a later paper.

The influence of body weight upon assimilation was twice observed; namely, the absence of glycosuria on 200 gm. of lung, with fat and sometimes 75 or 100 gm. of bread up to November 4, 1916, at weights below 10.5 kilos, and the occurrence of glycosuria a month later on 200 gm. of lung and 100 gm. of suet at a weight of 11.3 kilos; likewise the lower tolerance of early January, 1918, at a weight of 10.8 kilos, as compared with a month preceding at a weight of 9 kilos. The weight was finally allowed to fall very low, with a view to demonstrating a still higher tolerance, and then ultimately bringing back diabetes by fattening; but this plan could not be carried out because of the accidental death.

This dog was characterized throughout by oliguria and high renal thresholds for sugar. There is a question of the possible existence and injury of renal calculi from the outset, but the appearance of the kidneys did not suggest a very long standing trouble. Similar peculiarities of function have been observed in other dogs under similar conditions, and the conclusions regarding diabetes are supported by blood as well as urine analyses.

The experiment also served as a test of the specific influence of pre-formed carbohydrate as a cause of injury to the assimilation in dia-

betes. An animal was chosen with potentially severe diabetes, produced by removal of $\frac{1}{2}$ of the pancreas and demonstrated by the occurrence of glycosuria on December 4 to 8, 1916, on a diet of only 200 gm. of lung and 100 gm. of suet, when the body weight had, as stated, been built up to a level which was still subnormal. Under these conditions the dog was undernourished by means of a mixed diet containing an appreciable quantity of carbohydrate, and the experiment was continued long enough to test whether carbohydrate would prove itself a specific poison to the islands of Langerhans. The actual result was a marked gain in tolerance. This is best seen by comparison of the above mentioned glycosuria in December, 1916, on 200 gm. of lung and 100 gm. of suet at a weight of 11.3 kilos, with the tests a year later when 300 gm. of bread could be taken without glycosuria at a weight of 10.8 kilos, and when the glycosuria which was produced by the addition of glucose cleared up (January 4, 1918) on 300 gm. of lung and 100 gm. of suet. It may be concluded that the tolerance created by undernutrition was genuine, and that the feeding of carbohydrate within the limits of this tolerance did not damage the assimilation or the islands of Langerhans.

Apart from the above details, the main point of the experiment was to determine whether downward progress is inevitable in an animal with potentially severe diabetes, so that symptoms must ultimately occur in spite of dietary restriction. This dog was kept for approximately $1\frac{3}{4}$ years. Besides the gain in tolerance during this time, the intact state of the islands furnished additional evidence that no injurious change was in progress, and the indications are that the same condition of health could have been maintained indefinitely.

EXPERIMENTAL STUDIES ON DIABETES.

SERIES I. PRODUCTION AND CONTROL OF DIABETES IN THE DOG.

5. VARIOUS FAILURES OF DIETETIC TREATMENT, AND THEIR CAUSES.

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PLATES 65 AND 66.

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Glycosuria Controllable Only by Fatal Undernutrition, Because of Severity of Diabetes.

Dog B2-28.—Male; mongrel; black and white; age 1 year; slightly thin; weight 11 kilos. Dec. 18, 1913. Removal of pancreatic tissue weighing 32 gm. Remnant about main duct estimated at 2 gm. ($\frac{1}{17}$). Glycosuria of 0.55 per cent followed the operation, but diminished, and by Dec. 23 was absent. Continued attempts were then made to nourish the animal with low protein-fat diets, often subdivided into several meals during the day, but traces of glycosuria recurred each time, requiring further fasting and undernutrition. Feb. 24, 1914. The dog was at the point of death from undernutrition; weight 6.8 kilos; killed for autopsy.

Autopsy.—The pancreas remnant, not obviously sclerosed, weighed 1.2 gm.

Microscopic Examination.—Portions of the remnant showed interacinar pancreatitis, other portions normal parenchyma. The islands generally were few and small but not to the extent of a positively pathological reduction. Appearances of vacuolation were rare and doubtful, but search through many sections revealed a very few distinct examples.

Dog B2-29.—Male; mongrel; white; age $1\frac{1}{2}$ years; good condition; weight 10.6 kilos. Dec. 22, 1913. Removal of pancreatic tissue weighing 27.7 gm. Remnant about main duct estimated at 2.4 gm. ($\frac{1}{12}$ – $\frac{1}{13}$). Only faint glycosuria followed the operation. The subsequent undernutrition was more rigid than in Dog B2-28, in the sense that no more than a trace of glycosuria on a single day was permitted at any time. The same inability to develop a tolerance for any living diet was encountered, and by Apr. 7, 1914 the weight had declined to 5.9 kilos. On that day the dog was killed.

Autopsy.—The pancreas remnant, somewhat nodular and atrophic, weighed 1.65 gm. The liver appeared fatty, presumably from the high proportions of fat in the diet. The autopsy was otherwise negative except for emaciation.

Microscopic Examination.—There was moderate fatty infiltration in the liver; no noteworthy changes in the spleen, kidneys, adrenals, testes, thyroid, parathyroids, and hypophysis. The pancreatic parenchyma mostly showed inter-acinar fibrosis, in the form of light bands traversing it irregularly and often distorting acini. The fibrosis was presumably the result of damage from an acute inflammation following operation; there were no signs of recent or progressive changes. Islands were scarce and small to a markedly pathological degree. In some considerable areas free from fibrosis there was this same scarcity of islands, and the sections were made up of almost unbroken expanses of acini. No vacuolation was visible.

The pancreas remnants in these animals were small, and there was apparently further destruction of islands by inflammation. Diabetes was more or less completely suppressed by fasting and undernutrition, but as neither dog became able to tolerate a diet sufficient to support life, they both died in a little over 2 months of inanition. Very slight hydropic changes were demonstrable in the islands in the first dog, which had shown frequent traces of glycosuria, but none were found in the second dog, in which glycosuria had been more rigorously controlled. These examples are comparable with a few human cases of great severity, especially when the treatment consists in continual attempts to push the diet to the limit of tolerance.

Originally Mild Diabetes; Death from the Undernutrition Made Necessary by Prolonged Slight Overfeeding.

Dog B2-71.—Female; mongrel; yellow; age 3 years; good condition; weight 14.7 kilos. June 3, 1914. Removal of pancreatic tissue weighing 19.2 gm. Remnant about main duct estimated at 2.25 gm. ($\frac{1}{5}$). No glycosuria ensued on meat diet. On change to bread and soup, there was the usual single day of glycosuria, thereafter none. The following tolerance tests were performed while the animal was at a fairly constant weight of 13 to 13.5 kilos, no food being given on the test days.

June 26. Subcutaneous injection of 42 gm. of Merck anhydrous glucose in 30 per cent solution (3 gm. per kilo on basis of normal weight of 14 kilos). No glycosuria. July 8. Subcutaneous injection of 56 gm. of Merck glucose in 30 per cent solution (4 gm. per kilo on basis of 14 kilo weight). No glycosuria. July 17. Subcutaneous injection of 84 gm. of Merck glucose in 30 per cent solution (6 gm. per kilo on basis of 14 kilo weight). Glycosuria 0.3 per cent in 10 cc.

of urine; thereafter negative. July 30. 56 gm. of Merck glucose in 30 per cent solution given by stomach tube (4 gm. per kilo on basis of 14 kilo weight). No glycosuria. Aug. 12. 84 gm. of Merck glucose in 30 per cent solution given by stomach tube (6 gm. per kilo on basis of 14 kilo weight). 3 hours after dose, moderate sugar reaction in 1 cc. of urine from bladder; next morning trace in 420 cc.

The subsequent record is contained in Table I.

TABLE I.
Dog B2-71.

Date.	Body weight.	Diet.	Remarks.
1914	kg.		
Aug. 21-23	13.8	Bread and soup with 100 gm. of glucose daily.	No glycosuria.
" 24-26	13.9	Bread and soup with 200 gm. of glucose daily.	" "
" 27- Sept. 26	14-11	Bread and soup with 300 gm. of glucose daily.	Glycosuria varying from heavy to negative; generally slight. Diarrhea; decline of weight and strength.
Sept. 27- Oct. 27	11-12	Bread and soup <i>ad libitum</i> .	Occasional traces of glycosuria, governed by variations of appetite, increasing with the slight gain in weight.
Oct. 28- Nov. 7	12.1-11.8	Bread and soup first with 50, then with 100 gm. of glucose daily.	Heavy glycosuria, diminishing as appetite failed.
Nov. 8- Jan. 2, 1915	11.8-13.8	Bread and soup with a little meat and suet daily.	Glycosuria absent at first, then irregular traces, becoming heavy at end with gain in weight.
1915			
Jan. 3-6	13.8-13.1	Fasting.	Glycosuria absent.
" 7- Mar. 28	13.5-12.6	Beef lung <i>ad libitum</i> .	" "
Mar. 29- July 2	12.6-10.8	1 kilo of beef lung daily.	No glycosuria except on days of carbohydrate feeding, as recorded in Paper 2,* which showed tolerance between 100 and 200 gm. of bread or rice.
July 3- Aug. 3	10.8-12.5	Beef lung <i>ad libitum</i> with 50 gm. of suet daily.	No glycosuria except in occasional experiments, which showed that tolerance was less than 50 gm. of bread.

* Allen, F. M., *J. Exp. Med.*, 1920, xxxi, 399.

The dog was then used for experiments unrelated to the present subject. Prior to death on Nov. 24, 1915, glycosuria was beginning to appear on a carbohydrate-free diet of beef lung and suet.

Autopsy.—The important feature of the autopsy was the pancreas remnant, which weighed 4.1 gm.

Microscopic Examination.—The islands were unusually large and numerous, and contained a slight sprinkling of maximally vacuolated cells, evidently related to the overfeeding preceding death.

The record represents downward progress during approximately a year and a half of life after operation. The animal was never subjected to any heavy continuous glycosuria. Certain fluctuations of tolerance were related to changes of weight, as noted in Table I, but there was no obesity and the weight remained always below the original normal level. The dog was merely kept fed as close as possible to the limit of tolerance, up to January, 1915, on bread, thereafter on meat, and tests or experiments were frequently interpolated producing slight or transitory glycosuria. Slow decline of assimilation thus occurred notwithstanding hypertrophy of the pancreas remnant and its richness in islands.

This finding, together with a number of animal and human observations, suggests a functional injury or defect in the islands when their number and size seem sufficient to prevent diabetes. The degree of vacuolation as usual corresponded to the glycosuria.

The chief point in the experiment is its close imitation of human cases in which the diet is forced to the verge of tolerance and the patient commits occasional indiscretions causing transitory glycosuria. The familiar downward progress of such cases is accurately reproduced.

Dog B2-31 (Fig. 4).—Female; bull-terrier; white; age 2 years; good condition; weight 11 kilos. Dec. 23, 1913. Removal of pancreatic tissue weighing 19 gm. Remnant about main duct estimated at 2.6 gm. ($\frac{1}{3}$ – $\frac{1}{2}$). The dog was able to live on bread and soup with only slight intermittent glycosuria. Her gluttony and fondness for sugar made diabetes possible, as described in Paper 2.¹

Mar. 8, 1914. Fasting was begun. Mar. 12. Glycosuria was absent. Nevertheless, the fast was continued through Mar. 16. Mar. 17. The body weight was 8 kilos, and very low weighed diets were begun, generally 50 gm. of lung and

¹ Allen, F. M., *J. Exp. Med.*, 1920, xxxi, 382.

50 gm. of suet, reducing the weight to 7.4 kilos on Mar. 25. These diets also were given in the form of meals spaced as far apart as possible, in the hope of better utilization from the standpoint of diabetes and also of the protein economy in the sense of Thomas,² though no plain evidence of the benefit of such a course was ever seen. Beginning Apr. 2, the daily ration was increased to three meals of 30 gm. of lung and 30 gm. of suet each. By Apr. 24 the weight had risen to 9.3 kilos, and the diet was increased to 40 gm. of lung and 40 gm. of suet thrice daily.

Apr. 27, 4 p.m. A tolerance test was performed by subcutaneous injection of 11 gm. of Merck anhydrous dextrose (1 gm. per kilo on the basis of the normal weight of 11 kilos) in 30 per cent solution. 10 p.m. Urine by catheter was 128 cc. with 0.9 per cent glucose. Apr. 28, a.m. Urine by catheter was 388 cc. with 0.4 per cent glucose; *i.e.*, an excretion of 2.7 gm. and utilization of 8.3 gm. of the dose.

Glycosuria was then absent till May 1, when the body weight was 9.9 kilos. It was continuous for 3 days, May 1 to 3, but not above 0.4 per cent. After fast days on May 3 and 4, the same diet was resumed, with glycosuria absent except as follows: May 7, 0.2 per cent, stopped by a fast day; May 17, 0.3 per cent, stopped likewise; May 24, 0.5 per cent, stopped by 2 fast days. After this the diet was reduced to 20 gm. of lung and 30 gm. of suet thrice daily. Nevertheless, on May 30, at a body weight of 9.75 kilos, there was a return of 0.17 per cent glycosuria. Though this ceased within 24 hours, the fast was continued for 5 days.

Thereafter, on a diet of 30 gm. of lung and 30 gm. of suet thrice daily, there was a gradual gain of weight up to 10.8 kilos on July 3, when there appeared glycosuria of 0.46 per cent in 600 cc. of urine, increasing to 0.97 per cent in 835 cc. of urine on July 4.

Fasting was imposed till July 7, when the weight was 10.3 kilos. Thereafter 100 gm. of lung were fed daily. Though the weight fell slightly on this lower caloric diet, there was continuous slight glycosuria on July 11 to 13, not above 0.66 per cent. The diet was then changed to 100 gm. of fresh beef pancreas daily, and the same glycosuria (not above 0.7 per cent) continued till July 20, again illustrating the uselessness of pancreas feeding.

After a 4 day fast, the weight was down to 9.3 kilos on July 24, and the diet of 100 gm. of raw pancreas was resumed, together with 30 gm. of bone-meal, which was commonly used for such experiments, as previously stated. In addition to the usual purpose of preventing diarrhea and supplying salts to the body, the bone-meal might perhaps serve in this connection to neutralize gastric juice. July 28. There began an excretion of 1.5 per cent glucose in 425 cc. of urine, increasing to 4 per cent in 360 cc. of urine the next day. Fasting then stopped the glycosuria in 2 days, but was continued to Aug. 4, when the weight was 8.5 kilos. The diet of 100 gm. of pancreas was then resumed, with resultant glyco-

² Thomas, K., *Arch. Physiol.*, 1910, suppl., 249.

suria of 0.2 to 0.4 per cent on Aug. 6 to 8. Fasting was then instituted for 4 days. As it seemed evident that the rigorous undernutrition was beginning to be effective in diminishing glycosuria, a change was made to lung diet, to guard against ascribing any benefit incorrectly to the pancreas feeding. Accordingly, beginning Aug. 11, 100 gm. of lung were fed daily, with absence of glycosuria except for a trace on 1 day. The weight at this time was 8.15 kilos.

Aug. 18. Bread and soup were given by mistake, with resultant glycosuria of 6.6 per cent in 1,275 cc. of urine. Fasting till Aug. 25 was necessary before glycosuria ceased. Thereafter on a diet of 20 gm. of lung and 20 gm. of suet thrice daily, glycosuria was absent except for a trace on 1 day.

Sept. 11. As the dog had tired of fat, the diet was changed to 50 gm. of lung thrice daily. Sept. 13. Glycosuria of 1 per cent appeared, the higher protein as usual having a greater immediate effect than the higher calories. Sugar excretion was continuous on this diet, and by Sept. 26 had risen to 6 per cent. Accordingly, fasting was begun on Sept. 27; glycosuria was absent on Oct. 4; nothing more than 60 gm. of suet was given daily till Oct. 9, when a diet of 50 gm. of lung and 50 gm. of suet was begun, and increased on Oct. 16 to 80 gm. of lung and 80 gm. of suet. The weight was then 6.7 kilos.

Slight glycosuria, not above 0.5 per cent, occurred on Oct. 22, 23, 28, 29, 30, and Nov. 6. Suet was then omitted, but similar sporadic traces continued till Nov. 23, when 2 fast days were imposed, followed by a diet of 50 gm. of lung and 30 gm. of suet. The weight rose slightly, to 6.9 kilos on Dec. 9, on which day glycosuria of 0.6 per cent appeared.

With 2 days of fasting, the urine was sugar-free on Dec. 11. Dec. 12. A diet of 50 gm. of lung was begun, with the addition of 30 gm. of suet after Dec. 18. About this time the previously lively animal began to fail seriously. The digestion began to be impaired, as usual in cachexia, and by Dec. 31 the weight was down to 5.8 kilos. The diet was changed to 50 gm. of raw pancreas and 30 gm. of suet, in the hope of improving digestion, but the pancreas was of no avail even for this purpose. Liberal feeding with pancreas and fat was attempted on Jan. 2 and 3, 1915; the result was a glycosuria of 1 per cent on Jan. 4, while the weight had fallen to 5.3 kilos. The dog was chloroformed.

Autopsy.—The urine in the bladder was 13 cc., containing 0.53 per cent glucose, a trace of albumin, and no acetone. Except for emaciation and corresponding atrophy of viscera, the autopsy was negative as usual. The weights of the principal organs were as follows: liver 101 gm.; both kidneys 48.8 gm.; both adrenals 2 gm.; pancreas remnant 3.8 gm. The pancreas remnant was soft, lobulated, and in places semitranslucent.

Microscopic Examination.—The liver showed a considerable sprinkling of glycogen granules by Best's carmine stain; these were located in the periphery of the lobules, the centers containing none; fat was scanty. The acinar tissue of the pancreas was normal. Islands were remarkable for both fewness and smallness. Their cells were normal in appearance and no vacuolation was discoverable.

Notwithstanding the extreme emaciation and prostration, glycosuria evidently continued till death. The point is mentioned because dogs of this type herein correspond to the rule for human cases. In some totally depancreatized dogs authors have reported cessation of glycosuria before death.

In spite of the severity of diabetes thus indicated, no sign of hydropic degeneration was visible in the islands of Langerhans. Study of a sufficient series of animals gives a simple explanation. The overstimulation causing such visible degeneration is intense and the cellular disintegration rather rapid. The diabetes in this dog had been largely controlled by diet. Life had continued for more than a year, and an active hydropic process must have destroyed all the islands before this time. The milder degree of overstimulation and longer clinical course should naturally give rise to a slower island destruction, in the form of occasional loss of cells, which would seldom be demonstrable microscopically. This supposition, which in animals can be fully verified by observations of all types, degrees, and stages of the process, serves to explain the similar findings in human cases.

The principal reason for the detailed record given is to show the exact similarity to the clinical course of many human cases. The animal started with mild diabetes; in fact, the large size of the pancreas remnant and the subsequent hypertrophy would probably have permitted a cure had opportunity been given. Gluttony and carbohydrate excess made the diabetes severe. A familiar plan of treatment was then employed, in the form of a protein-fat diet pushed to the point of maintaining the highest possible weight and strength. There was the usual onset of glycosuria with gain in weight, the necessity for repeated fasting periods, and the gradual decline of tolerance, weight, and strength. This occurred without any breaking of diet on the part of the subject. The two principal features of cases under such management are thus illustrated; first, life and strength are preserved much longer than when active diabetic symptoms are allowed to continue; second, the alleged "spontaneous" downward progress of human patients occurs in typical slow but sure form.

Glycosuria Uncontrollable after Protein Overfeeding.

Dog B2-56.—The record of this animal was given in Paper 3³ as an example of downward progress on excessive protein diet. The attempt to control the condition by fasting beginning May 14, 1914, gave results shown in Table II.

The cachexia was unusually rapid in progress, and even before the fast the dog had lost considerable hair and was developing ulcers at points of bony pressure, especially over the ischiatic tuberosities and

TABLE II.

Dog B2-56.

Date.	Urine.		Remarks.
	Volume.	Glucose.	
1914	cc.	per cent	
May 14	840	1.7	Weight 13.5 kilos. Fasting.
" 15	490	3.0	Fasting.
" 16	530	3.5	"
" 17	415	1.6	"
" 18	450	1.0	"
" 19	360	1.5	"
" 20	267	1.4	"
" 21	397	1.5	"
" 23	430	1.3	"
" 24	535	2.4	Weight 9.9 kilos. Diet of meat <i>ad libitum</i> begun because of dangerous weakness.
" 25	1,000	0.7	Diet of meat <i>ad libitum</i> .
" 26	1,150	0.8	" " " " "
" 27	690	0.6	" " " " "
" 28			Weight 9.7 kilos. Moribund. Killed for autopsy.

the joints of the limbs. These were foul and spreading in character, without tendency to heal. The one at the right elbow perforated into the joint and was probably the origin of the large axillary abscess of creamy pus found at autopsy. It is unknown why a few partially depancreatized dogs show cachexia and susceptibility to infection almost like totally depancreatized animals. The "gangrene" and lowered resistance of human patients are fully reproduced in such animals. Also in occasional human cases the diabetes is evidently too severe to be controlled by fasting.

³ Allen, F. M., *J. Exp. Med.*, 1920, xxxi, 560.

Glycosuria from Prolonged Protein-Fat Overfeeding, Checked at First by Short Fasting, Later Uncontrollable.

Dog B2-57.—Female; mongrel; yellow; age 5 years; good condition. May 4, 1914. Received at normal weight of 11 kilos. After reduction of weight to 8.65 kilos by fasting, on May 21 partial pancreatectomy was performed; 19.6 gm. of tissue removed, and remnant left estimated at 3 gm. The dog was used to test the effects of fat feeding and obesity. The first continuous glycosuria began Dec. 24, at a weight of 16.3 kilos. This persisted till stopped by fasting, Jan. 4 to 6. The record for 1915 is given in Table III.

Dog B2-30.—Female; mongrel; yellow; age 1½ years; good condition; weight 10 kilos. Dec. 22, 1913. Removal of pancreatic tissue weighing 19.5 gm. Remnant about main duct estimated at 2.5 gm. Glycosuria was absent after operation till milk was fed on Dec. 26. It then remained heavy (up to 3.3 per cent) on bread and soup diet till Jan. 1, 1914, after which a large (unweighed) admixture of glucose was required to produce glycosuria. The dog gradually reached the point of refusing the mixture absolutely; therefore on Jan. 17 the diet was changed to chopped meat mixed with lactose, which was eaten abundantly and maintained glycosuria as high as 5 per cent. After Jan. 22 heavy glycosuria continued on a diet of meat only. Feb. 2. Fasting was begun in the attempt to stop glycosuria (Table IV).

Blood Transfusion.—Feb. 20. Transfusion was undertaken partly in an attempt to increase strength and partly to determine whether, when everything possible was done by fasting, there might be any transfer of pancreatic hormone in the blood sufficient to check the diabetes. 4.30 p.m. Urine under the cage was 25 cc., containing glucose 4.8 per cent. When all was ready for transfusion, catheterization yielded 2 cc. of urine, with glucose 6.2 per cent. A large needle was then inserted into an external jugular vein, and by the use of vaseline-coated syringes without anticoagulants blood was drawn from a normal dog and injected into this dog to the amount of 350 cc. by 5.30 p.m. At this time the dog showed collapse, passed urine and feces, and seemed on the point of death, from toxic action of the infused blood or simple overfilling of the circulation. The transfusion was therefore ended, and the emptiness of the bladder verified with the catheter. The total urine for the transfusion period was 3 cc., containing 6.4 per cent glucose. 6.15 p.m. The urine obtained by catheter was 4 cc., containing 3 per cent glucose and slight albumin. The dog drank thirstily. 8.30 p.m. The urine by catheter was 19 cc., with a faint trace of albumin and 1.7 per cent sugar. 2.30 a.m. The urine by catheter was 26 cc. with 2.5 per cent glucose and the same faint albumin. The mere traces of glycosuria on the succeeding days, as shown in Table IV, may appear striking, but the writer is inclined to interpret them as accidents of renal permeability rather than benefit to the diabetes.

TABLE III.

Dog B2-57.

Date.	Body weight.	Diet.	Urine.	
			Volume.	Glucose.
1915	kg.		cc.	per cent
Jan. 1	16.3	100 gm. of lard and 1,000 gm. of beef lung, the latter not all eaten.	650	1.5
" 2		100 gm. of lard and 1,000 gm. of beef lung, the latter not all eaten.	880	4.2
" 3		Most of food uneaten.	890	1.6
" 4		Fasting.	180	0.7
" 5	15.4	"	339	0.22
" 6		"	261	0
" 7	14.8	"	113	0
" 8	14.5	"	56	0
" 9	14.3	100 gm. of lung.	196	0
" 10		200 " " "	194	0
" 11	14.0	300 " " "	231	Doubtful.
" 12	14.13	400 " " "	280	0
" 13	13.73	500 " " "	203	0
" 14	13.55	500 " " "	186	0.18
" 15	13.60	600 " " "	239	1.37
" 16	13.75	700 " " "	107	1.33
" 17		Fasting.	81	0
" 18	13.30	"	65	0
" 19	13.20	500 gm. of lung.	119	0.7
" 20	13.05	500 " " "	369	0.23
" 21	12.86	Fasting.	165	Doubtful.
" 22	12.60	"	154	0
" 23	12.45	400 gm. of lung.	250	0.20
" 24		400 " " " 30 gm. of suet.	386	2.50
" 25	12.40	Fasting.	14	0.90
" 26	12.18	"	99	0.60
" 27	12.06	"	95	Very faint.
" 28	11.90	"	50	0
" 29	11.75	100 gm. of lung.	170	0
" 30	11.75	150 " " "	350	2.80
" 31		Fasting.	273	2.00
Feb. 1	11.30	"	142	1.50
" 2	11.15	"	211	0.80
" 3	11.10	"	195	1.00
" 4	10.85	"	185	0.70
" 5	10.68	"	81	0.60
" 6	10.61	"	89	2.00
" 7		"	183	1.25

TABLE III—*Concluded.*

Date.	Body weight.	Diet.	Urine.	
			Volume.	Glucose.
1915	kg.		cc.	per cent
Feb. 8	10.25	Fasting.	176	3.00
" 9	10.10	"	204	2.78
" 10	9.90	"	185	2.00
" 11	9.80	"	70	2.00
" 12	9.50	"	116	1.20
" 13	9.50	50 gm. of glidine.	295	3.03
" 14		Fasting.	135	1.40
" 15	9.30	"	102	1.70
" 16	9.15	"	105	2.50
" 17	9.00	"	175	2.50
" 18	8.77	40 gm. of extract of beef.	385	2.53
" 19	8.78	Fasting.	274	1.33
" 20	8.85	50 gm. of lard; 50 gm. of meat.	260	1.92
" 21		50 " " " 50 " " "	120	1.41
" 22		50 " " " 50 " " "	219	0.72
" 23	8.25	50 " " " 50 " " "	570	0.30
" 24	8.10	50 " " " 50 " " "	800	0.69
" 25	8.10	50 " " " 50 " " "	752	0.72
" 26	7.85	50 " " " 50 " " "	747	0.80
" 27	7.80	50 " " " 100 " " "	1,295	0.90

Subsequent History of Dog B2-30.—After Mar. 21, the dog was given 25 gm. of lung and 25 gm. of suet two or three times daily; *i.e.*, a diet of either 50 or 75 gm. each of lung and suet. Apr. 2. At a body weight of 5.5 kilos, glycosuria of 2.7 per cent appeared, and was stopped only by fasting till Apr. 5. The diets thereafter were lower than before, sometimes only 10 gm. of lung and 10 gm. of suet thrice daily. With advancing cachexia the digestion became poor; glycosuria was absent, but by Apr. 21 the weight had fallen to 4.3 kilos, and the animal could barely stand. Accordingly, on that date it seemed necessary to give four meals of 40 gm. of lung each, without suet. The result was an immediate glycosuria of 1.7 per cent in 140 cc. of urine. Though the diet was increased to 50 gm. of lung four times daily, which was as much as the dog would take, glycosuria ceased through failure of digestion. Apr. 24. The animal was at the point of death and was chloroformed.

Autopsy.—Performed immediately. Aside from the extreme emaciation and accompanying atrophy of viscera, the autopsy was negative. The pancreas remnant, normal in appearance and consistency, weighed 3.9 gm.

Microscopic Examination.—The acini were normal, uneven in the degree of filling, but mostly containing abundant zymogen. Island tissue was scarce

TABLE IV.
Dog B2-30.

Date.	Body weight.	Urine.		Dextrose-nitrogen ratio.
		Glucose.	Nitrogen.	
1914	kg.	gm.	gm.	
Feb. 2	8.6	9.25		
" 3				
" 4	8.1			
" 5	7.8	1.25		
" 6	7.8	2.21		
" 7	7.8	3.42	3.14	1.09
" 8		0.92	3.11	0.29
" 9	7.5	1.48	2.81	0.53
" 10	7.25	0.60	2.69	0.22
" 11	7.15	4.45	3.25	1.37
" 12		2.22	2.67	0.83
" 13	6.90	0.06	2.60	
" 14	6.80	Faint.	1.93	
" 15		"	2.48	
" 16	6.55	0.64	3.40	
" 17	6.50	1.02	2.08	0.49
" 18	6.30	0.62	1.80	0.34
" 19	6.20	2.83	1.73	1.63
" 20	6.08	3.68	2.44	1.51
" 21	6.40	Faint.	2.43	
" 22	6.23	"	2.63	
" 23	6.02	4.13	3.82	1.08
" 24		7.32	4.99	1.47
" 25	5.65	2.82	3.53	0.80
" 26	5.60	Faint.	3.41	
" 27	5.43	0	4.66	
" 28	5.45	0	3.30	
Mar. 1	5.35	0	4.44	
" 2	5.20	0	3.01	
" 3	5.18	0	3.07	
" 4	5.20	0	2.42	
" 5	5.38	0	2.74	
" 6	5.28	0.90	3.14	
" 7	5.33	0.24	2.20	
" 8	5.28	Very faint.	2.28	
" 9	5.20	0	1.31	
" 10	5.15	0	1.98	
" 11	5.23	0	2.06	
" 12	5.23	0	1.54	
" 13	5.20	0	1.87	
" 14	5.20	0	1.97	
" 15	5.28	0	1.89	
" 16	5.30	0	1.97	
" 17	5.25	0	1.89	
" 18	5.28	0	2.11	
" 19	5.20	0	2.75	
" 20	5.20	0	2.20	

almost to the vanishing point. Search revealed occasional tiny clumps of cells of island character. No Bensley stains were made, but by comparison with other animals in which such stains were made these surviving, non-vacuolated cells may be interpreted as alpha cells, in confirmation of the original observations by Homans.⁴ In rare instances such cell clumps contained one to three maximally vacuolated cells, presumably the last remains of degenerated beta cells. As usual, the islands were not replaced by fibrosis; the tissue merely gave the impression of unbroken expanses of acini.

Starting with sugar-free urine on January 1 on bread and soup diet, this dog showed a downward progress which was so rapid that within 1 month the pancreatic function had fallen too low to support life.

The dextrose-nitrogen ratio was never maximal and was generally low. It is known from the literature that even totally depancreatized dogs do not necessarily show the full 2.8 ratio during fasting. Partially depancreatized dogs of the present type seldom or never show this full ratio during fasting, yet the absence of it is no proof that life can be maintained.

The occasional temporary cessation of glycosuria in this animal during fasting is not unusual under these circumstances and is presumably due to changes of renal permeability. It is probable that the blood sugar remained high till about the time of death.

Transfusion of blood improved the strength slightly and perhaps altered renal permeability temporarily, but had no positive effect upon the diabetes. In view of the similar result in a child,⁵ it may be concluded that the effect of transfusion is negative.

The evidence of the hopelessly low pancreatic function was that with the lowest possible diets and body weights, lasting freedom from glycosuria could not be achieved, so that the animal necessarily died from weakness.

The extreme degree of exhaustion and disappearance of island tissue correspond to the lowered function as mentioned.

⁴ Homans, J., *J. Med. Research*, 1914, xxx, 49; 1915, xxxiii, 1.

⁵ Allen, F. M., Stillman, E., and Fitz, R., Total dietary regulation in the treatment of diabetes, Monograph of The Rockefeller Institute for Medical Research, No. 11, New York, 1919, Chapter III, Case 45, 361.

Dog B2-01 (Figs. 2 and 3).—Part of the history of this animal was given in Paper 3. The terminal period so closely resembles that of Dog B2-30 that it can be summarized here very briefly, particularly because it supplements the

TABLE V.

Dog B2-01.

Date.	Urine.						Plasma sugar.	Remarks.
	Volume.	Glucose.		Total ni- trogen.	Dextrose- nitrogen ratio.	Nitroprus- side reaction.		
1919	cc.	per cent	gm.	gm.			per cent	
Oct. 22	145	2.20	3.19	4.66	0.68	Heavy.	0.432	Weight 13.3 kilos. Fasting.
" 23		Heavy.				"		Fasting.
" 24	155	1.80	2.79			"		"
" 25		Heavy.				"		"
" 26	65	2.29	1.48			"		"
" 27		Heavy.				"		"
" 28	118	1.15	1.36			"		"
" 29	50	0.95	0.48	0.98	0.49	Moderate.		"
" 30	110	0.45	0.50			"		"
" 31	200	Moderate.				"		"
Nov. 1	200	0.18	0.36	1.72	0.21	Slight.		"
" 2		Slight.				"		"
" 3		Faint.				"		"
" 4		0				"		"
" 5		0				Negative.	0.322	Fed 50 gm. of tallow.
" 6		0				"		" 100 " " "
" 7		0				"		Refuses fat. Fed bones.
" 8	1,500	0.80	12.0	5.6	2.1	Negative.		Fed 100 gm. of beef.
" 9	270	0.55	1.5	3.2	0.47	"		Fasting.
" 10	190	0.25	0.48			Slight.		"
" 11	275	Slight.		1.40		Moderate.		"
" 12	200	"		1.30		Slight.		"
" 13		"				Negative.		"
" 14	210	0.40	0.84	2.03	0.41	"		"
" 15		Slight.				"		"

other by its blood sugar analyses, which were lacking in Dog B2-30. Heavy sugar and acetone reactions being found present on the diet of lung and suet as stated,⁶ and the blood plasma on Oct. 16, 1919 showing 0.520 per cent sugar and a slight nitroprusside reaction, fasting was begun on this date in the

⁶ Allen,³ p. 570.

attempt to control the diabetes, though with little expectation of success. After Oct. 22, the seriousness of the condition being confirmed by the stubborn persistence of glycosuria, occasional quantitative analyses of sugar and nitrogen were performed (Table V). Urine was voided without catheterization, and the analyses apply to such daily specimens, not to accurate 24 hour specimens.

Nov. 15, p. m. The dog was dying. An intraperitoneal saline injection given after taking blood at 8 p.m. failed to benefit. 10 p.m. Animal found dead. The results of blood analyses are shown in Table VI.

This animal was one of those in which the severity of diabetes is too great to be checked by fasting. This condition is more common in experimental than in clinical diabetes, but is by no means unknown in the latter. The greater susceptibility of human patients to acidosis

TABLE VI.

Dog B2-01.

Date.	Plasma sugar.	Blood urea per 100 cc.	Plasma chlorides per 100 cc.	Plasma CO ₂ capacity.	Plasma nitroprusside reaction.
<i>1919</i>	<i>per cent</i>	<i>mg.</i>	<i>mg.</i>	<i>vol. per cent</i>	
Oct. 16	0.520	23.4	550	59.8	Moderate.
" 22	0.432				Slight.
Nov. 5	0.322	26.0	533	56.6	Negative.
" 14	0.624	71.4	562	62.4	Slight.
" 15, 10 a.m.	0.872				Negative.
8 p.m.	0.600	38.6	536	59.4	"

is the probable reason why, with hopelessly severe diabetes, they generally die quickly in coma rather than in the prolonged cachexia which is characteristic of dogs. It will be shown later that under suitable conditions dogs also reach a state where they develop coma with either feeding or fasting.

It is not known whether the D:N ratio in this animal was ever maximal. The high ratio of 2.1 on November 8, in consequence of only a single feeding of 100 gm. of beef, suggests that the full 2.8 ratio might have been present on feeding; but on the other hand, the unusual glycosuria on this date may have represented partly a mere sweeping out of retained sugar. The most striking feature is the remarkably low sugar excretion and D:N ratios in a hopelessly severe case of diabetes. Such submaximal ratios have been the rule in other dogs of this sort during fasting.

Renal impermeability, which is a familiar phenomenon in many human patients, seemed to be largely responsible for the low and variable glycosuria. Absence of glycosuria, on November 4 to 7, with plasma sugar as high as 0.322 per cent, was similar to what has been observed in several other animals under similar conditions, and could not be interpreted as indicating that the diabetes was under control. It may be noted that this impermeability existed in an animal without histological signs of nephritis at autopsy, and with no known cause of impaired renal function other than the diabetes.

The apparent renal impermeability did not include retention of chlorides so far as indicated by the blood analyses. The blood urea rose to a strikingly high figure on November 14, but fell before death in a manner difficult to explain by simple retention. The observations concerning urea also closely reproduce the conditions found in certain human patients.

Nitroprusside reactions were heavy in the urine and slight in the plasma on beginning fasting, and diminished during the fast, as usual in human patients. This clearing of the acetone bodies was approximately parallel with the decline of glycosuria. These tests became negative during the period of freedom from glycosuria, and returned after glycosuria had been restored by protein feeding. They were negative preceding death, as often happens in cachexia. The principal point of these observations is that in this hopelessly diabetic animal the sugar, whether retained from renal impermeability or any other cause, seemed somehow to be used in a way to prevent acetoacetic acid formation.

*Dog C3-56 (Fig. 9).—*Female; mongrel; black and white; age 3 years; moderately well nourished; weight 15.3 kilos. Jan. 27, 1916. Received. Raised a litter of pups in laboratory. July 13. Removal of pancreatic tissue weighing 35 gm. Remnant about main duct estimated at 4.6 gm. (about $\frac{1}{3}$). Glycosuria occurred on bread and soup feeding, but later required glucose for its continuance. The tolerance was thus broken down so that by the middle of Aug. glycosuria was present on a diet of 1 kilo of beef lung. High fat diets were then given, for purposes and with results explained in detail in a previous publication.⁷ The table there shown carried the record up to Nov. 4. The condition then was one of persistent and stubborn hyperglycemia without glycosuria,

⁷ Allen, F. M., *Am. J. Med. Sc.*, 1917, cliii, 349, 364.

such as excessive fat diets often produce in human patients. The utmost effort was then made to save the animal by diets such as those used for human cases. The diet of 200 gm. of lung daily was continued to Nov. 18, with the purpose of sparing body protein and conserving strength as well as possible, while building up tolerance by undernutrition. On Nov. 18 the plasma sugar was still 0.208 per cent, though the weight had fallen to 7.6 kilos, and the animal was weak. It was therefore deemed necessary to reduce the protein, and the diet was changed to 75 gm. of lung and 75 gm. of suet, with the addition of one raw egg daily to contribute variety and supply any possible need for fat-soluble vitamins. As the appetite failed, other forms of cooked and raw meat were substituted for variety in the same quantity. By Dec. 3 all food was refused. The urine remained free from sugar and acetone as it had been throughout this entire period, but the plasma sugar of the moribund animal was still 0.137 per cent and the carbon dioxide capacity 37.6 volumes per cent, with analyses for all three acetone bodies negative and all other signs of acidosis absent. The dog then weighed 7.25 kilos in a condition of extreme cachexia, with spreading and perforating ulcers on the legs suggestive of diabetic "gangrene." The animal was therefore killed for autopsy.

Autopsy.—The emaciated cadaver contained no visible fat. The liver was small, not fatty, weighing 218 gm. The kidneys, pale and edematous appearing, weighed together 56.5 gm. The viscera were otherwise negative except for atrophy. The pancreas remnant, normal in appearance and consistency, weighed 4.25 gm.

Microscopic Examination.—The organs were negative except for the usual vacuolation of renal tubules due to glycogen or fat. The pancreas remnant, free from fibrosis, consisted of almost unbroken expanses of small acini, some entirely empty but the majority containing zymogen. Only the last remains of exhausted islands were found, in the form of small clumps of cells, sometimes maximally vacuolated (presumably beta cells), sometimes non-vacuolated (presumably alpha cells), sometimes a mixture of the two. There was also vacuolation in the cells of some of the small ducts, as sometimes found in this extreme terminal stage of diabetes.

The experiment illustrates hyperglycemia and impairment of assimilation brought on chiefly by fat feeding, and downward progress notwithstanding absence of glycosuria. Conditions in many human cases are thus closely imitated.

The hydropic degeneration of islands with hyperglycemia without glycosuria is also illustrated. Under these as under all other circumstances, the tendency is for canine diabetes to run a more rapid course than human diabetes. For this reason hydropic degeneration is always easier to demonstrate in dogs, but may be assumed to occur

more slowly in human patients who show a similar but slower downward progress with hyperglycemia.

The clinical and anatomic findings combine to support the view that the internal pancreatic function was here too deficient to support life. A similar conclusion may be drawn from similar evidence in some of the worst human cases.

One point worth mentioning is that diabetic dogs are not necessarily wretched and cachectic. Especially those possessing a maximum of pancreatic tissue, and best suited for therapeutic and some other tests, may be normal appearing and also comfortable and happy. The other point of importance is the degree to which the health and life of these animals is dependent on their diet. These facts are shown more plainly by photographs (Figs. 1 to 12) than by any verbal description, and also the parallelism with the conditions of human patients is more clearly illustrated.

CONCLUSIONS.

1. Practically every detail of clinical diabetes can be reproduced in partially depancreatized animals. The resemblance is made still more exact by the susceptibility of such dogs to acidosis and coma, as will be shown in later papers, and also by the similarity of the anatomic changes in the islands of Langerhans. These animals are therefore useful test objects for a therapeutic investigation.

2. These animals at first show considerable tendency to regain assimilation, comparable to that in the early stages of most human cases of diabetes; and in some instances they recover so as to be able to endure any degree or duration of carbohydrate feeding and can be made diabetic only by removal of additional pancreatic tissue. Similar recovery in some human cases, especially after acute pancreatitis, is a probability. This recuperative tendency can be negated by overfeeding, even without glycosuria. With duration of the diabetes the power of recuperation diminishes and practically disappears in dogs as in human patients.

3. In the absence of progressive pancreatitis or other extraneous causes, these dogs show no inherent downward tendency in their assimilation. This conclusion rests upon observations as long as 6

years from the first pancreas operation and 3 years of known diabetes. This absence of inherent progressiveness is what should naturally be expected in animals with simple resection of part of an organ, and serves further to fit them for accurate feeding experiments.

4. Every detail of the downward progress of human patients on various diets is reproduced in such animals. They lose assimilation and die most rapidly on diets rich in carbohydrate, and less rapidly on excess of other foods. The differences between sugar and starch, and between starch and protein, seem to be only those of degree and time rather than anything absolute. The important point is that, granting the absence of spontaneous downward tendencies as stated, all the different kinds and degrees of downward progress in the records of animals in this and the preceding paper are purely the results of overstrain of the internal pancreatic function by excess of food.

5. Varying degrees of success and failure in the dietetic control of diabetes are also illustrated. The benefit of the classical treatment by exclusion of preformed carbohydrate and limitation of protein is confirmed, in the prolongation of life and well-being to some extent in nearly all cases and perhaps indefinitely in some of the mildest cases. In the great majority of cases such a therapeutic result is not permanent, and downward progress is finally observed if the observations are continued long enough. In most of these cases life, strength, and assimilation can be preserved for a much longer time by a degree of undernutrition suited to the severity of the diabetes, and accomplished by limitation of fat in the diet. The permanence of such control is supported by the unimpaired or rising assimilation in experiments of 1 to $1\frac{3}{4}$ years duration, but still longer observations would be desirable. Diabetes of great severity is controllable only by correspondingly radical undernutrition. In still more severe cases glycosuria can be abolished only by a degree of undernutrition which entails final death from inanition. In the most severe cases glycosuria cannot be stopped, evidently because the assimilative power is too low to dispose of even the minimum supply of food materials; namely, that derived from the body stores in fasting.

6. A claim of saving every patient, no matter how near death, would be a preposterous one for any remedy in any disease, and the

animal experiments do not support such a claim for diabetes. Also it is unreasonable to expect the actual cure of an organic deficiency by diet, and the diet treatment in animals just as in patients generally represents the sparing rather than the restoration of the weakened function. The basis of the belief in the inherently progressive tendency of severe cases of clinical diabetes is shaken by the exact reproduction of such case histories by diet in animals which are free from spontaneous downward tendencies; but there is still lack of a sufficient number of patients treated on the principle of relief of the total metabolic burden to demonstrate the absence of such inherent progressiveness in human diabetes. As described elsewhere,⁸ the principle mentioned has given encouraging results in proportion as it has received actual application in practice. The clinical problem requires the same prolonged careful control of all discoverable symptoms as in animals, and cases too severe for such control, or complicated with infections, violations of diet, etc., are on the same plane as animals in which a similar condition has been produced. Some proportion of cases, especially in young persons, will be found suitable for accurate determination of the question of whether all or most cases of severe diabetes are inevitably progressive and hopeless, and the writer looks forward to publishing such a series. Irrespective of the outcome, the raising of this question is justified by its importance. On the theoretical side, it involves both the general prognosis and the nature of the process underlying diabetes. On the practical side, it is already established that diet is at least the chief cause of downward progress, and it is important to eliminate this cause by avoiding the dietary injuries illustrated in the animal experiments.

⁸ Allen, F. M., Stillman, E., and Fitz, R., Total dietary regulation in the treatment of diabetes, Monograph of The Rockefeller Institute for Medical Research, No. 11, New York, 1919.

EXPLANATION OF PLATES.

PLATE 65.

FIG. 1. Dog B2-00. The picture was taken while the animal was diabetic in 1917, but shows approximately the condition existing during the 6 years of laboratory life and still maintained on the restricted protein-fat diet.

FIG. 2. The usual sturdy appearance of Dog B2-01 at her ordinary weight between 13 and 14 kilos.

FIG. 3. Dog B2-01 in July, 1917, when fattened with suet to 16.5 kilos and with hyperglycemia present.

FIG. 4. Dog B2-31 in the state of moderate undernutrition which is indicated by the distinctness of individual vertebrae along the back, and which was effective in abolishing glycosuria for several months. The dog had first become diabetic with $\frac{1}{3}$ - $\frac{1}{2}$ of the pancreas through her gluttony for bread and sugar. Afterward, though undernutrition was maintained, it was not thorough enough. Diets of protein and fat were pushed too close to the verge of tolerance, raising the weight not to normal, but nevertheless higher than the assimilative power could carry. The final result was hopeless diabetes.

FIG. 5. Dog B2-25 in the state of emaciation which had to be maintained for a number of months to control diabetes with only $\frac{1}{12}$ to $\frac{1}{13}$ of the pancreas present. At this weight of 11 kilos the animal remained strong and lively, and required restraint to keep him quiet for photographing. Through hypertrophy of the pancreas remnant it became possible gradually to raise the weight to 17 kilos, or only 1 kilo below normal.

PLATE 66.

FIG. 6. Dog B2-79; control to Dog B2-56. The pancreas remnant was $\frac{1}{8}$, and microscopically showed more inflammation than that of Dog B2-56. The normal weight of 15 kilos was reduced to 11.7, in which condition 200 to 300 gm. of bread were requisite for glycosuria. The animal is shown 8 months after operation, when a diet of 1 kilo of lung had raised the weight to 15.5 kilos, and glycosuria was accordingly present. The dog was kept in good condition and subjected to several other fluctuations of weight and tolerance, till death 17 months after operation.

FIG. 7. Dog B2-80 at a time when glycosuria had been brought on by fattening to 18.2 kilos on a diet of beef lung and suet. The animal was specially valuable because of excellent digestion due to the large remnant ($\frac{1}{4}$ - $\frac{1}{3}$ of the pancreas) with which diabetes occurred. The tolerance was high at weights as low as 13.2 kilos. She was used for various observations during 9 months, and the persistent high fat feeding finally brought on not only glycosuria but fatal acidosis. The dog was as sleek and healthy looking as this when she went into typical coma at a weight of 17.25 kilos. Animals of this sort give the closest reproduction of clinical acidosis.

FIG. 8. Dog C3-27 1 week before death in coma, with intense glycosuria, acidosis, and lipemia present; the dog wags her tail, but she was already nauseated and depressed, and the dry nose is a further sign of ill health. The obesity maintained by the high fat diet is evident.

FIG. 9. Dog C3-56 in the closing period. After the damage of high fat diet, the animal was reduced by undernutrition to a feeble state; but though glycosuria was abolished, hyperglycemia was persistent up to death from cachexia. The similar weakness and deficiency of assimilation of certain human patients under similar conditions are well known.

FIG. 10. Dog C3-86 in moderate undernutrition. The fluctuations of tolerance in parallel with the weight were previously described.⁹ The dog was vigorous and lively through $1\frac{3}{4}$ years from the time of operation to death.

FIG. 11. Dog D4-28, $1\frac{1}{4}$ years after an operation which had left $\frac{1}{8}$ of the pancreas. The normal weight was 12 kilos. After prolonged undernutrition, the weight here had been gradually built up to 10.6 kilos. The dog was observed for $1\frac{3}{4}$ years after operation, and remained lively and symptom-free until death from urinary calculus. One feature of the experiment was 50 gm. of bread in the regular ration, showing that a dog with the marked degree of potential diabetes represented by such a small pancreas remnant could by undernutrition be enabled to tolerate such a quantity of carbohydrate for such a length of time without sign of injury.

FIG. 12. Dog D4-52 in Jan., 1918, $\frac{1}{2}$ year after the operation which created mild diabetes. The susceptibility to glycosuria and lowering of assimilation on carbohydrate diet were first demonstrated, and then observations were begun to test whether, with this mild degree of diabetes, a liberal carbohydrate-free diet (with restriction of protein to 500 gm. of beef lung) could be tolerated indefinitely.¹⁰

⁹ Allen,⁷ Chart 8.

¹⁰ Allen,³ pp. 564-573.





RELATION OF THE PORTAL BLOOD TO LIVER MAINTENANCE.

A DEMONSTRATION OF LIVER ATROPHY CONDITIONAL ON COMPENSATION.

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PLATES 67 TO 71.

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In the course of observations on the rôle of the liver in blood formation and destruction, we have had occasion to ligate portal branches to the organ. The ensuing changes have been of such striking character as to merit study for their own sake; and the present paper is concerned with them. There already exists, of course, a considerable literature on so obvious a theme. For the moment it may suffice to state in this connection that according to the generally accepted view occlusion of a portal branch to the liver has no effect on the organ save when a grave derangement of the systemic circulation is also present. The complete local parenchymal atrophy that in our experiments regularly followed such occlusion was unforeseen, as was the further observation that the atrophy is conditional, being dependent upon a compensatory hypertrophy of the remainder of the organ.

Method.

The liver of the rabbit is singularly adapted for experiments involving the blood vessels and bile ducts, since it consists of two separate masses, each with its own vessels and ducts. The rabbit may indeed for operative purposes be said to possess two livers. They are of very unequal size, the larger, or main liver, as we shall call it, formed of the left anterior and posterior lobes and the right anterior lobe with the gall bladder, being three times as big as the smaller, or lobe mass, which consists of the right posterior and caudate lobes. The lobe mass contains just enough parenchyma, as Ponfick¹ showed, to suffice for the

¹ Ponfick, E., *Virchows Arch. path. Anat.*, 1889, cxviii, 209; 1890, cxix, 193.

needs of the organism when the main liver is ablated. Through it, under such circumstances, all of the portal stream finds room to pass.

In our experiments the portal trunk to the main liver of the rabbit has been ligated just above the caudate lobe. By such means the whole portal stream is diverted to the lobe mass. The caudate lobe, though a part of the latter by reason of its parenchymal connection with the right posterior lobe, has an added source of venous blood through a small branch arising from the portal trunk at the level of the ligature and frequently compromised by it. To avoid the irregularity thus introduced, the caudate lobe was tied off and cut away as a routine measure. A piece of the tissue was sectioned to determine the condition of the liver.

In ligating the portal trunk great care was taken not to interfere with the main bile duct and hepatic artery. The operation was carried out under ether on rabbits of from 1,400 to 2,300 gm. Closure was done in three layers. Occasionally a fatal necrotic process spread from the ligated caudate stump, but the great majority of the animals recovered without complication and remained in perfect health. They were killed with chloroform at periods of from 12 days to 6 months after the original operation. The liver masses were weighed separately after the blood, as yet unclotted, had flowed away from the severed vessels. This, it was felt, would result in a truer estimate of relative amounts of parenchyma than if the blood were retained by closing the vessels, as was Ponfick's method. According to Ponfick,¹ the normal liver of the rabbit averages 3.56 per cent of the gross body weight, though he prefers to use 4 per cent in calculations. On his estimation the main mass forms 74.7 per cent of the total, the right posterior lobe 19.3 per cent, and the caudate lobe 6 per cent. In fifteen normal rabbits we have obtained an average figure of 3.45 per cent for the liver's proportion of the body weight, with variations ranging from 2.18 per cent to 5.25 per cent. The main mass averaged 72.3 per cent of the total, the ablated caudate 4 per cent, and the right posterior lobe with the caudate stump 23.7 per cent.

Early Changes after Local Portal Occlusion.

The immediate results of diverting the stream are striking.

Within a minute or so the main liver becomes much smaller, of a deeper purple, and flaccid, whereas the isolated lobe, which now receives all the venous blood, is swollen, tense, and of a rather bright red. In animals dying after 1 or 2 days hemorrhages into spleen, stomach, and small intestines are sometimes found, such as Ponfick observed after removal of the main liver; but, as he also noted, these are infrequent in vigorous animals. The acute passive congestion responsible for them is quickly relieved as the stream bed in the lobe mass is widened through the hypertrophy of the latter.

The mass receiving the portal blood begins to hypertrophy within 3 days by cell proliferation within the lobules, as after ablation of the main liver,² and by the end of 12 days the tissue has usually more than doubled, and after 15 days may have trebled. Subsequently its bulk increases more slowly, but eventually reaches that of the entire original liver, and usually surpasses it. Concurrent with the hypertrophy is a progressive atrophy of the mass deprived of portal blood (Figs. 1 and 2).

Owing to reduced capillary distension the lobuli fall together to some extent immediately after the ligation, and within the next few days their cells, which now appear crowded, are noted to have grown smaller, especially near the central vein where the blood supply is poorest. If the animal is weak and dies early, a marked local widening of the capillaries may here be noted, with fine, brown pigmentation of the parenchyma. The condition then is identical with that known in human pathology as the "atrophic red infarct of Zahn." In vigorous rabbits the capillary widening is slight, often absent, and the intralobular atrophy alone attracts attention. The liver mass sometimes dwindles within 12 days to about one-half its original bulk (Table I), though usually the change is slower. Its lobes are flabby, wrinkled, purple, and at this particular period their surface is usually mottled with ill defined, slightly raised, pale spots which may be half a centimeter in diameter (Fig. 3). Several lobuli or parts of them are included in such areas. They are well seen only on the capsular surface, are not degenerative in character, become much more prominent when the blood has partially escaped from the tissues, and are probably areas of relative anemia. The general condition of the liver at this time has much in common with that in dogs during the period of adjustment after an Eck fistula.^{3,4} But outspoken degenerative changes are rare in the rabbit liver as compared with the dog, though sometimes a moderate, central fatty degeneration may be noted. The tissue when cut is soft, dark purple, and spleen-like; the lobuli are very small and indistinct. Bile ducts, blood vessels, and interlobular connective tissue are all rendered unusually prominent by the dwindling in parenchyma, and, simulating trabeculae, add to the spleen-like appearance of the tissue.

Microscopically, one finds many small lobuli to a field, and there is a great increase in the number of cells per unit of surface (Fig. 1), so that the nuclei appear crowded. These last, as well as the cytoplasm, have greatly decreased in bulk, though otherwise they appear unchanged. At the periphery of the lobules a few endothelial cells along the capillaries may be somewhat swollen

² Ponfick, E., *Virchows Arch. path. Anat.*, 1895, cxxxviii, suppl., 81.

³ Whipple, G. H., and Hooper, C. W., *Am. J. Physiol.*, 1917, xlii, 544.

⁴ We wish to thank Dr. G. H. Whipple for sections illustrative of the Eck fistula liver.

with granules of a light brown, iron-containing pigment. Such pigmented Kupfer cells become more prominent as time goes on. There is no absolute increase in connective tissue.

Late Changes after Local Portal Occlusion.

The changes up to this point have been partially described by Steenhuis.⁵ In his most advanced instance, an animal killed 4½ months after the portal ligation, a further moderate increase in pigmentation and atrophy was noted, but nothing more, a fact difficult to understand save on the assumption that portal collaterals to the main liver had developed, or that the animal was old or in poor condition, all of which factors largely affect the changes. For the atrophy goes further, and quickly too, resulting in a disappearance of all the parenchyma of the main liver, a process sometimes practically completed within 2 months, as we shall show.

Between the 12th and the 40th day after the portal ligation a circulatory readjustment occurs in the dwindling liver mass. Pale spots are no longer seen on its surface, which is of a brighter, more normal red, though tinged with brown. The organ cuts with difficulty, owing to the survival of all its ducts, vessels, and connective tissue, which are brought nearer together by the disappearance of parenchyma; and the tissue disclosed by the knife is more markedly spleen-like, with no trace of a lobular pattern. Histologically, the parenchyma may seem like the normal at first sight, except for the very small size of lobuli and cells, a marked irregularity in the arrangement of the former, and perhaps some irregular capillary widening. Fatty changes are entirely absent, and the atrophy is more evenly distributed. On close scrutiny one perceives here and there parenchymal elements almost without cytoplasm and with small pycnotic nucleus. These scattered cells in the last stages of disappearance are somewhat more numerous toward the center of the lobules. The cell cords in general may be especially atrophic here, and their capillaries wider than at the periphery, additional indications that the central parenchyma suffers most. Pigmented Kupfer cells, distended to a spherical or egg shape, are increased in number, but are found as before only near the periphery of the lobule. The interlobular tissue has nowhere invaded the parenchyma, is absolutely unincreased, and new formed bile ducts are not present. The ducts and vessels, unchanged from their original size, are bent into convolutions as the mass grows smaller, so that their number seems multiplied on cross-section (Fig. 5).

⁵ Steenhuis, T. S., *Experimenteel en kritisch onderzoek over de gevolgen van poortaderafsluiting*, Proefschrift Rijks-Universiteit te Gröningen, 1911.

The period required for complete disappearance of the parenchyma varies with the individual (Table I). When the animal is opened the stomach is found to lie in the concavity of the diaphragm, and only on lifting it away is the insignificant remnant of the main liver discovered, with a gall bladder of normal bigness sessile upon it (Fig. 9). The compensating lobe, of great size, extends far down over the abdomen. On nearer inspection, the original lobes of the main liver are found to be represented by three little, flabby, pinkish brown tags (Fig. 4). Cultures from these are sterile. Their surface is roughened by numerous, close packed and tortuous vessels and ducts. Sometimes red varices stud the surface here and there. The hepatic veins, of almost the normal size, at once attract attention as distended cords, grossly disproportionate to the tissue out of which they spring. The hepatic artery, too, is still large. On section the tissue is extremely tough and shows no parenchyma, but everywhere the gaping mouths of vessels and ducts in a slight matrix of connective tissue. The blood immediately above the ligature on the portal trunk is usually fluid, and thrombi are always absent from the branches of the vein.

Microscopically, a few liver cells can be found even when the changes are most pronounced, for the reason that there exist in practically every case a few minute, collateral venules bringing portal blood to the tissue. The rapidity and completeness of the atrophy is in our experiments proportionate to the number of these little collaterals. Their influence may be directly seen where they enter the liver. For example, in Rabbit 27, killed 118 days after the ligation, 2 minute venules were found coursing to small masses of healthy parenchyma in the midst of the atrophy (Fig. 4).

The final changes have much interest. Little by little the lobular units grow smaller and more irregular in form, so that a central vein, when discoverable, may be far off to one side (Fig. 5). There is still no connective tissue invasion or proliferation, but Glisson's capsule becomes increasingly prominent in the picture, appearing to close in on the lobules and envelop them. As the parenchyma grows less, so do its attendant capillaries disappear, and they never survive when the liver cords are gone. Soon there remain only scattered islands consisting of a few small, but healthy looking liver cords with the characteristic capillaries, set in a matrix of connective tissue (Fig. 7). Here and there, near by, three or four parenchymal cells may perhaps be found as an isolated cord, not infrequently with a capillary along one side; and liver cells separated by the en-

veloping matrix of connective tissue may still be recognized. Many such isolated cells have lost their characteristic ground glass appearance, stain a clear pink with eosin, and may be of blunt spindle shape with a relatively large pyknotic nucleus (Fig. 8). Finally parenchymal elements become rare (Fig. 10). Their situation is sometimes indicated by the irregular zone of Kupffer cells distended with pigment that mark the border of the original lobule. As the atrophy increases such elements become prominent, and rounded nests or aggregations of thirty to forty are not infrequent (Fig. 6). Always they are confined to the region of the original periphery of the lobules and are separated from the parenchyma only by the disappearance of the latter. The total absence of invasive tendencies on the part of the connective tissue could not be more clearly shown than by this fact.

The final tag of ducts, vessels, and connective tissue (Fig. 10), representing 50 to 70 gm. of main liver, weighs, in the absence of coccidiosis, cirrhosis, or other intercurrent proliferative change, only 1 to 1.8 gm., which may perhaps be taken as nearly representing the original weight of the non-parenchymal elements. If a cirrhosis was originally present as shown by the caudate sections, the surviving tissue is of greater bulk.

Conditional Character of the Atrophy.

The liver atrophy in the dog and in man following diversion of the entire portal stream through an Eck fistula is never great. Hence we have questioned whether the complete atrophy observed on local portal diversion in rabbits is inevitable or dependent upon hypertrophy elsewhere. To test the matter hypertrophy has been largely prevented in some animals by tying the bile duct to the lobe mass of the liver after diverting the entire portal stream to it as usual. Under such circumstances the lobe mass undergoes some increase in size through cell proliferation, but combined with this is a continuous, scattered biliary necrosis, and by the end of 12 days a diffuse cirrhosis makes its appearance. After 25 to 30 days the tissue, though still of greater bulk than normal, is indurated and shows microscopically an almost complete replacement with connective tissue. The changes will be more fully described in a later paper. The fact to be emphasized here is that in the absence of hypertrophy of the lobe mass the main liver fails to undergo marked atrophy, although deprived of the portal stream. Such slight atrophy as occurs may be looked upon as inevitable to the circulatory change as such.

The rabbits used were kept under identical conditions and had approximately the same weight. In one series the portal blood was diverted from the main liver and the caudate lobe ablated as usual, while in the other ligation of the bile duct or ducts to the lobe mass was also performed. The local bile stasis had caused no jaundice, and the animals were in good health. The ducts to the posterior lobe, for there may be two or three, lie in an exposed position and can readily be isolated and tied off without damage to their surroundings. From this circumstance, taken with the differing results obtained in the two series of animals, it follows that direct nerve injury can be ruled out as a cause for the complete atrophy after simple portal occlusion. Animals with livers originally abnormal were discarded.

A complicating factor made necessary the early comparison of the series. The progressive cirrhosis occurring when the bile duct from the lobe mass is ligated brings about after a time an obstruction to the portal flow, with chronic passive congestion of the viscera. The spleen enlarges greatly, sometimes becoming cylindrical; and venous collaterals appear rapidly, as a rule preventing marked ascites, though this was once noted. None of the new venous channels had importance for us save such as might enable the portal blood to regain its old stream bed in the main liver; but unfortunately some of this character frequently developed within a few weeks. Usually they followed the course of the veins of Charpy, but sometimes found a way through adhesions. Portal diversion alone not infrequently led to their development in small number. The majority of the rabbits were killed and autopsied prior to their appearance; that is, 12 to 15 days after operation. Such small collaterals to the main liver as were then observed have found place in the general record (Table I). All the animals were killed soon after a feeding.

The weight of the entire liver was somewhat below the normal average in five animals killed 12 days after simple portal occlusion—3.22 per cent of the gross body weight as compared with a normal of 3.45 per cent. Every individual weight, though, was within the normal range of 2.18 to 5.28 per cent. In the five rabbits with local bile stasis added to portal ligation, whereby parenchymal destruction was superimposed upon hypertrophy, the livers weighed more, averaging 3.62 per cent of the gross weight. In the animal with simple atrophy, and its companion with bile stasis, killed after 15 days, the weights were 4.5 and 5.78 per cent of the gross, respectively. The average after longer periods was 3.73 per cent for ten rabbits killed from 21 to 68 days after portal diversion, and 3.69 per cent for three animals with an additional bile stasis, examined after from 21 to 30 days. It would seem that the reparative changes went beyond a mere replacement, as

TABLE I.
Liver Changes Following Diversion of the Portal Stream to the Lobe Mass.

Rabbit No.	Body weight.		Collateral veins to liver.	Liver weight.			Liver's percent of body weight.			Condition of main liver.	Remarks.
	At operation.	Final weight.		Entire mass.	Main liver.	Lobe mass.	Entire mass.	Main liver.	Lobe mass.		
Duration of experiment, 12 days.											
1	2,200	2,475	None.	72.4	28.3	44.1	2.93	1.15	1.78	Marked atrophy; many pale spots.	
2*	2,050	2,175	One of 1½ mm.	78.6	43.3	35.3	3.62	1.99	1.63	Little atrophy; no spots.	
3	2,150	2,050	One of ¾ mm.	75.4	39.8	35.6	3.68	1.94	1.74	Marked atrophy; many spots.	
4	2,150	2,050	One of 2¼ mm.	76.6	48.0	28.6	3.74	2.34	1.4	Little atrophy; no spots.	
5	1,900	1,600	One of 3 mm.	46.4	21.7	24.7	2.9	1.36	1.54	Marked atrophy; no spots.	
6	1,925	1,600	Two of 1 mm.	65.2	30.2	32.8	4.07	1.89	2.05	Little atrophy; no spots.	Of total liver weight 2.2 gm. is caudate stump in simple hypertrophy.
7		1,525	One of 1 mm. and several smaller.	48.8	16.8	32.0	3.2	1.1	2.1	Marked atrophy; some spots.	Animal has lost little if any weight.
8	1,850	1,700	None.	55.0	29.9	25.1	3.24	1.76	1.48	Marked atrophy; many spots.	
9	1,550	1,275	One of 1½ mm.	43.1	23.2	19.9	3.38	1.83	1.55	Marked atrophy; many spots.	Animal grew thin after operation.
10	1,750	1,650	One of ½ mm.	56.4	38.3	18.1	3.42	2.32	1.1	Slight atrophy; some spots.	

Duration of experiment, 15 days.

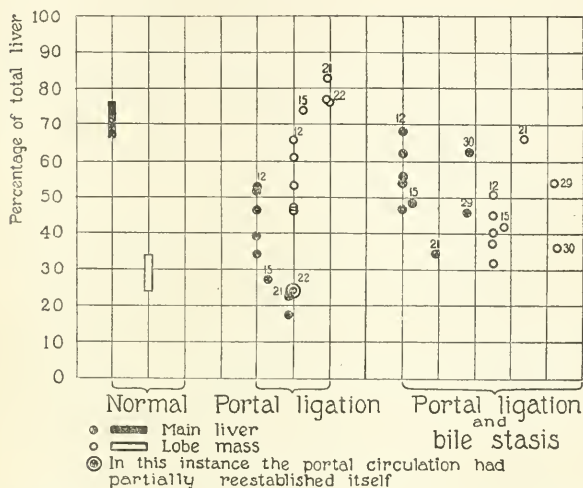
		Later periods.										Of total liver weight 7.8 gm. is caudate stump in simple hypertrophy.
		1,425	1,525	One of 1 mm.	70.2	18.6	51.6	4.6	1.22	3.38	Marked atrophy; some spots.	
11		1,425	1,525	One of 1 mm.	70.2	18.6	51.6	4.6	1.22	3.38	Marked atrophy; some spots.	Of total liver weight 7.8 gm. is caudate stump in simple hypertrophy.
12		1,425	1,400	Two of 1 mm.	80.9	38.9	34.2	5.78	2.78	2.44	Slight atrophy; no spots.	
		Later periods.										
days		2,300	2,250	Only very fine ones.	73.5	17.0	56.5	3.27	0.76	2.51	Marked atrophy.	
13	21	2,300	2,250	Only very fine ones.	73.5	17.0	56.5	3.27	0.76	2.51	Marked atrophy.	At least 2 gm. of main liver consists of coccidial change.
14	29	2,175	2,300	Only very fine ones.	78.5	37.0	41.5	3.42	1.61	1.81	Slight atrophy.	Marked chronic passive congestion. Spleen weight 4.1 gm.
15	30	2,275	2,275	Numerous very fine ones; also one of 1 mm. and of 2 mm.	69.1	44.0	16.4	3.02	1.94	0.72	No atrophy.	Caudate stump of 8.7 gm. in simple hypertrophy gives a by-pass for the blood. No passive congestion.
16	22	1,825	1,900	One of 5 mm.	122.7	30.5	92.2	6.47	1.61	4.86	Moderate atrophy.	Portal flow to main liver almost wholly reestablished.
17	21	1,875	1,375	One of 2 mm.	68.7	22.8	45.9	5.0	1.66	3.34	Moderate atrophy.	Chronic passive congestion; ascites; marked loss of weight.
18	21	1,575	1,750	None (?).	55.4	9.3	46.1	3.17	0.53	2.64	Marked atrophy.	Collaterals not sought for.
19	21	1,675	1,950	None.	77.5	15.2	62.3	3.98	0.78	2.2	"	No passive congestion.
20	25			Not noted.	73.5	19.5	54.0				"	No passive congestion.

* The findings in bold faced type relate to animals in which the bile duct of the lobe mass was also ligated.

TABLE I—*Concluded.*

Rabbit No.	Body weight.		Collateral veins to liver.	Liver weight.			Liver's per cent of body weight.			Condition of main liver.	Remarks.
	At operation.	Final weight.		Entire mass.	Main liver.	Lobe mass.	Entire mass.	Main liver.	Lobe mass.		
Later periods.											
21	55	2,125	2,550	84.3	5.0	79.3	3.31	0.2	Advanced atrophy.	Unusual delay in atrophy. Liver weighed with blood retained.	
22	58	2,025	2,250	74.1	10.1	64.0	3.3	0.46	"		
23	64		2,150	59.9	6.7	53.2	2.79	0.31	"		
24	65		2,050	70.9	1.8	69.1	3.46	0.09	Practically complete atrophy.		
25	68	2,025	2,050	79.3	4.0	75.3	3.87	0.2	Almost complete atrophy.		
26	104	2,250	2,300	82.4	9.4(!)	73.0	3.58	0.41	Advanced atrophy.	Unusual delay in atrophy. Liver weighed with blood retained.	
27	118	1,825	2,375	99.0	1.4	97.6					
28	185		2,400	71.1	1.6	69.5	2.96	0.07	Complete atrophy.		

with other tissues. The idea is borne out by the decrease in size of the liver occurring later. In seven animals killed 55 to 185 days after portal diversion the liver averaged only 3.22 per cent of the body weight.



TEXT-FIG. 1. The change in relative proportions of main liver and lobe mass after local portal diversion with and without ligation of the bile duct of the mass receiving the portal stream (see Table I). The range of the normal proportions as observed in thirteen rabbits, is given in the short columns. The caudate lobe had not been ablated in these instances as in the case of the operated individuals. The results in the latter are given in dots, and the number of days elapsing after operation is indicated in small numerals. When there is but one of these above a vertical row of dots it is supposed to apply to all.

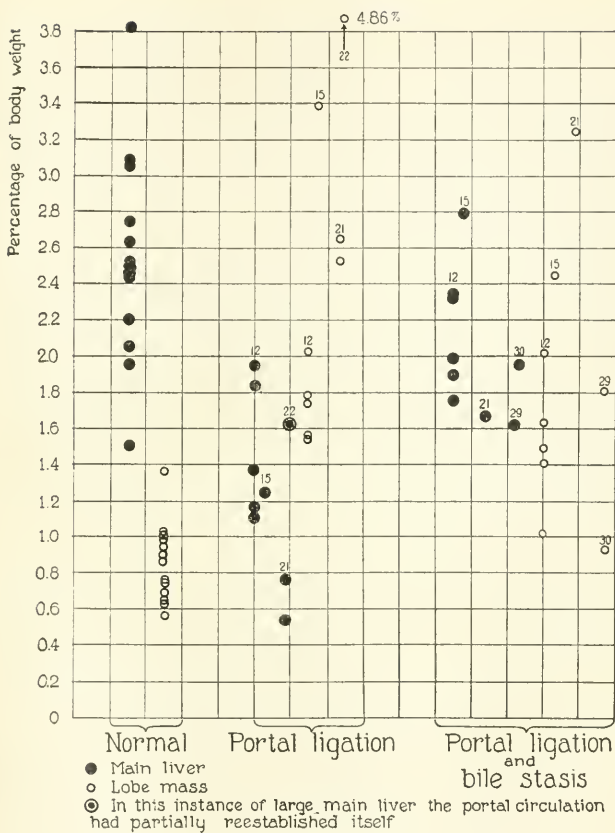
The distribution of the hepatic parenchyma was very different in the two series of animals (Text-fig. 1). Under normal conditions the relative size of the liver masses does not vary much, the larger ranging from 75.7 to 66.3 per cent and the smaller from 24.3 to 33.7 per cent of the whole. After operation not only did the general relations

change but the individual variations in the liver masses became greater. The latter alteration has no interest for us, however. The important point is that the main liver retained far more of its parenchyma after diversion of its portal blood if progressive hypertrophy of the lobe mass was prevented by biliary stasis (Text-fig. 2). The fact is illustrated by differences in both the proportional and actual weights of the liver masses; and the longer the period after operation the greater was the disparity noted, granting the absence of large hepatopetal collaterals. After 4 weeks of portal diversion plus bile stasis the main liver was sometimes three times as big as after but 3 weeks of uncomplicated portal diversion (compare Nos. 14 and 18, Table I, and see also Text-fig. 2).

The importance of portal venules measuring 1 to 2 mm. in diameter for the maintenance of the large mass of liver tissue that remains shortly after operation is negligible, as the general records show (Table I). The animal with the largest collaterals after 12 days, Rabbit 5, the subject of simple portal diversion, had a liver atrophy far greater than occurred in any of the controls with almost no collaterals but with bile stasis. Even at a late period of atrophy, when the liver is greatly shrunken, a collateral of 1 mm. diameter suffices to maintain but a small portion of parenchyma, as we have repeatedly found (Fig. 4). Yet the importance of very large collaterals is not to be gainsaid. It is well seen in the case of Rabbit 16, Table I.

A number of special instances excluded from the table and the text-figures for good cause might be quoted to illustrate the dependence of the pronounced liver atrophy on a compensating hypertrophy. One will suffice.

In a rabbit weighing 1,600 gm., killed 12 days after simple portal diversion, an unexpectedly large main liver was found. It weighed 33.2 gm., representing thus 2.1 per cent of the body weight of the animal, a proportion met with in other animals only when local bile stasis had prevented hypertrophy of the lobe mass. There were no collaterals to explain the condition, but in the lobe mass, which weighed only 22 gm., an atrophy of unknown cause was encountered, affecting at least half of its original tissue content, while the remainder was hypertrophied.



TEXT-FIG. 2. Changes in weight of the main liver and lobe mass, as expressed in percentages of the gross body weight. The number of days elapsing after operation is given in small numerals as in Text-fig. 1.

Functional Activities of the Atrophic Tissue.

The functional activities of an hepatic tissue deprived of portal blood and competing with a hypertrophic parenchyma that receives the entire portal stream have much interest. Asp⁶ showed long ago that the immediate effect of a local portal occlusion in the rabbit is to lessen secretion from the affected parenchyma. In our animals the atrophic tissue still secreted bile in good quantity at a period when the liver mass possessing a monopoly of the portal stream was far advanced in compensatory hypertrophy.

The biliary secretion of the rabbit is thin and copious—5 cc. per kilo of animal in 1 hour according to Heidenhain,⁷ or 169 gm. per kilo of liver. Krause⁸ obtained an average of 115.7 gm. of bile per kilo of animal in 24 hours. The weight of his animals was taken after the intestinal contents had been removed, which entailed a reduction of some 20 per cent from the gross weight. The quantity of rabbit bile varies greatly from hour to hour; the pigments fail to give the ordinary color reactions in a satisfactory way; and neither they nor the other bile constituents have been sufficiently studied to establish a norm. For these reasons we have merely observed the amount and color of the secretion from liver masses in atrophy and submitted it to Pettenkofer's test, while well aware that the latter gives positive results with other substances than bile salts, notably with cholesterol.

Method.

Two operations were necessary, the first to ligate the portal vein to the main liver, and the second, weeks or months later, for the ligation of the bile duct to the atrophic mass and the production of a fistula. The duct was twice tied above the point of entrance of the branch from the compensating lobe mass; the gall bladder was slit at its tip; a fine, rubber-covered catheter was introduced; and this last was sewed to the lip of the abdominal wound in such fashion that the atrophic liver was left in its usual position. The gall bladder and larger bile passages above the ligatures were gently and repeatedly flushed with salt solution injected and withdrawn through the cannula. This was done to wash out all traces of the bile previously reaching the gall bladder from the hypertrophic tissue. The washing never caused even a temporary cessation of the bile flow so far as could be judged from cases in which this was abundant. The cannula was now connected with a thick walled, rubber tube that led to a sterile, flat bottle strapped to the abdomen with adhesive. Through the stopper an air-vent tube was carried around to the animal's back under a many tailed bandage. The

⁶ Asp, G., *Arb. physiol. Anstalt Leipzig*, 1873, viii, 124.

⁷ Heidenhain, R., in Hermann, L., *Handbuch der Physiologie*, Leipsic, 1883, v, pt. 1, 252.

⁸ Krause, W., *Die Anatomie des Kaninchens*, Leipsic, 2nd edition, 1884.

bottle was adjusted at such an angle that the bile dropped into it from the end of the tube. The animals tolerated the arrangement well, and sometimes bile collection was continued for a period of 48 hours.

The several experiments in which bile collections were made from main livers in a state of moderate atrophy need not be cited in their entirety. In all an unexpectedly large amount of bile was obtained.

For example, 15 cc. of bright green bile giving a marked Pettenkofer reaction was obtained in 18 hours from an atrophic main liver weighing 19.5 gm., in the presence of a lobe mass of 54 gm. At the time when the bile fistula was produced, 25 days after portal closure, the original ligature on the vein was found to have relaxed, allowing a slight leak to the main liver, and accounting for the slightly delayed atrophy. A second, and completely occluding, ligature was laid on prior to the collection of bile. The latter was secreted at a rate much below the normal, though still considerable—43 cc. per hour per kilo of the atrophic tissue.

More interest attaches to instances in which the compensating mass approximated the whole original liver in size, and presumably in function, since the atrophic main liver had become very small.

Rabbit 24 had on the 65th day after operation a main liver weighing but 1.8 gm. and a compensating mass of 69.1 gm. On the day previous, while the animal was in excellent condition, the main bile duct was ligated and a fistula produced as usual. The ligature was placed above the entrance into the main duct of the branch from the left posterior lobe, and in consequence the secretion from only about two-thirds of the atrophic mass was obtained. At autopsy no portal collaterals to this fraction were found, nor was there the least biliary obstruction. The gall bladder was empty. From it 0.8 cc. of clear, watery fluid had come away in 23½ hours. This had a faint greenish tinge and gave a faint Pettenkofer reaction. The microscope later showed that definite islands of liver parenchyma were present in the tissue furnishing the bile.

A similar duct ligation with the branch from the left posterior lobe excluded was done in Rabbit 23 with a main liver of 6.7 gm., and 53.2 gm. of compensating tissue 64 days after operation. The atrophic tissue from which bile was collected, some two-fifths of the whole main liver, received a portal collateral 1.5 mm. in diameter. Nevertheless, there was secreted from it only 0.9 cc. of bile in 21 hours, though this was medium green in color and gave an outspoken Pettenkofer reaction. The tissue that was drained still consisted predominantly of parenchyma.

Rabbit 21 had a main liver of 5 gm. and a compensating mass of 79.3 gm. on the 55th day after operation. The main duct was ligated successfully, but a blood clot stopped the cannula, so the animal was killed 4 hours after the operation. During this period it had been lively. The stomach was full of food. Only 0.5 cc. of fluid, and this faintly green, was present in the collapsed gall bladder. No obstruction was found to the flow of bile through the ducts; and the

main liver mass was still predominantly parenchymal. A single hepatopetal portal collateral existed, 0.5 mm. in diameter.

The amount of parenchyma present in the atrophic main liver of Rabbit 24 was small. In the other two instances, though, where there was more of it, the amount of bile was far below that called for on calculation, had the conditions been normal, after allowing for 1.8 gm. of scaffolding, ducts, and vessels in the atrophic mass. Furthermore, the secretion in two of the three animals was markedly deficient in pigment, and in one gave but a faint Pettenkofer reaction. Yet there is no doubt that the hepatic tissue, even when extremely atrophic, does manufacture bile of a sort, and is not prevented from so doing by the presence of a compensatory liver mass of very large size. It should be remembered in this connection that the atrophic tissue receives through its large hepatic artery a liberal supply of blood.

Glycogen was sought with Best's carmine stain in three instances of far advanced atrophy. The method is subject to some errors, as Rusk⁹ has brought out, yet it seemed preferable to a chemical analysis, because of the greatly altered proportion of parenchyma in the tissue. The preparations showed a practically identical amount and distribution of glycogen in the hypertrophic and atrophic parenchyma of the same individual, even in cases as advanced as Nos. 24 and 25 (Table I). Neither the competition of the hypertrophic mass nor its favorable situation on the portal stream was sufficient to deprive the main liver of even relatively little glycogen. But this is scarcely surprising when one considers how widely the substance is distributed in the body, and that dextrose is normally present in the arterial blood. An interesting aspect of the findings is the evidence they give for the belief that such liver parenchyma as survives atrophy to a late period remains in remarkably good condition. For the glycogen content of unhealthy tissues is usually greatly altered.

DISCUSSION.

Conditional Atrophy in the Dog.

Recently one of us, with Dr. Philip D. McMaster, has ligated the portal trunk to the three upper lobes of the liver in a number of dogs. Changes ensued much more slowly than in the rabbit and are not yet

⁹ Rusk, G. Y., *Univ. California Pub. Path.*, 1912, ii, 83.

complete, after 3 months, but the tissue deprived of portal blood has diminished to less than one-third of its original bulk through a simple atrophy, with a corresponding hypertrophy elsewhere.

Review of the Literature.

Previous work on the result of local portal occlusion has been well summarized by Winternitz,¹⁰ who himself treats of the early changes in human livers. Though some observers, Solowieff¹¹ and others, have claimed that the occlusion of portal branches leads to cirrhosis, and Ehrhardt¹² stated that a moderate atrophy without cirrhosis ensues, the prevailing view, long since crystallized, is that no liver changes occur either in man or the laboratory animals unless pressure is abnormally low in the hepatic artery or high in the vena cava as the result of a disturbed systemic circulation. Under such contributing circumstances one finds within a few days the so called red infarct of Zahn in the region deprived of portal blood. Here the lobular capillaries are much distended, presumably from a venous stasis, and the liver cords somewhat atrophied. In the gross the liver portion is dark red and slightly sunken. The late changes have not been described, according to Winternitz, for the reason that such diseases as produce embolus or thrombosis in the portal system almost always end fatally within a brief period.

It is interesting to note here and there in the literature isolated statements that confirm our findings, and like them indicate that the prevailing view as just given is erroneous. Thus Frerichs¹³ stated in 1858 on the basis of his own observations that local portal occlusion leads to parenchymal atrophy with liver scarring. According to Ehrhardt,¹² Nauwerck saw a case in which the left lobe of the liver was diminished to the size of the fist, with compensatory hypertrophy of the right lobe, as a result of long standing occlusion of the left portal branch. Ehrhardt himself produced a moderate atrophy with compensatory hypertrophy by local portal ligation in cats, but he did not follow the changes long.

As already mentioned, most authors state that the development of a red infarct of Zahn after local portal occlusion is conditional upon a general circulatory disturbance. The fact that Zahn¹⁴ himself produced typical red infarcts by injecting mercury into a mesenteric vein of otherwise healthy dogs seems to have been forgotten, as has also his view that the condition is an atrophy from inactivity owing to the lack of portal blood, combined with a pressure atrophy

¹⁰ Winternitz, M. C., *Bull. Johns Hopkins Hosp.*, 1911, xxii, 396.

¹¹ Solowieff, A., *Virchows Arch. path. Anat.*, 1875, lxii, 195.

¹² Ehrhardt, O., *Verhandl. deutsch. Ges. Chir.*, 31 Kong., 1902, xxxi, 544.

¹³ Frerichs, F. T., *Klinik der Leberkrankheiten*, Brunswick, 1858 (Sydenham Society's translation, *A clinical treatise on diseases of the liver*, London, 1861).

¹⁴ Zahn, F. W., *Verhandl. Ges. deutsch. Naturforsch. u. Aerzte*, 69 *Versamml.*, 1897-98, ii, pt. 2, 10.

from stasis as the result of retrograde pressure in the hepatic veins. The drawing given by Chiari¹⁵ shows a histological condition identical with that present in our rabbits 12 to 15 days after the ligation. There can be no doubt that a general circulatory derangement renders the atrophy more prominent, as was the case in some of our animals that fell sick. The widening toward the central vein of the lobular capillaries, when marked, is doubtless often the result of retrograde pressure; but it occurred in moderate degree in some of our well conditioned rabbits and would seem then to have been merely the consequence of rapid atrophy of the liver cords. That there may occur complete parenchymal atrophy which is conditional on hypertrophy of the remaining parenchyma has not been realized heretofore.

Since the completion of our work the paper of Steenhuis⁵ has come to attention. It has attracted little notice among pathologists, owing perhaps to the fact that its author laid stress rather upon the surgical implications of his findings than upon the pathological. But Steenhuis ligated the portal trunk to the main liver of the rabbit, just as we have done, and observed an atrophy of medium grade thereafter, with the development of pigmented Kupffer cells. He did not follow the changes to even approximate completion, since in his most advanced instance a considerable bulk of liver tissue still remained, as the pictures and description clearly show. He noted the influence of portal collaterals to check the atrophy and drew the conclusion, since proved erroneous, that a direct portal stream is essential to liver survival.

Physiological Considerations.

Several reasons can be suggested for the changes which follow a local diversion of the portal stream. Among them are the following:

(a) *Direct Influence of the Altered Circulation.*—By the ligation of its portal trunk the main liver is supplied solely with arterial blood, which latter may be so unsuited to the liver cells that they can survive and function only in the absence of competition, as under the circumstances of an Eck fistula. The high oxygen content of the blood can scarcely be invoked as a cause of the hypothetical unfitness, since the atrophy is least at the periphery of the lobules where oxygenation is greatest.

(b) *Altered Functional Opportunities.*—By local portal obstruction one portion of liver tissue is deprived of its normal opportunity to obtain many substances and must compete with another receiving them in undue quantity. Functional atrophy and hypertrophy should follow as a matter of course. The extent and rapidity of the

¹⁵ Chiari, H., *Z. Heilk.*, 1898, xix, 475.

changes alone are surprising. The hypertrophy goes on almost, perhaps quite, as rapidly as if the tissue deprived of portal blood had been ablated.

Few rabbits survive the abrupt removal of the main liver. Ponfick's instances do not enable one to judge when the compensating hypertrophy was complete. Von Meister¹⁶ states that the right posterior lobe and caudate attain the weight of the whole liver in from 45 to 60 days. But this weight he puts at only 2.91 per cent of the gross body weight, whereas 3.45 per cent is nearer the truth and has been the basis of our calculations. In one of our cases such a proportion to the body weight was actually attained by the hypertrophied mass within 65 days and in another within 68, while the functional adequacy of the tissue was attested by complete atrophy of the main liver in one instance and approximately complete atrophy in the other. These results become more striking when one considers that our animals were adults weighing 2,000 gm., whereas von Meister's were young and of 900 to 1,400 gm., that is, far more favorable to hypertrophy, as he showed; and when the further fact is added that the caudate lobes were cut away in our cases so that there was less tissue capable of hypertrophy. Nasse¹⁷ found that 4 months was required for the disappearance of the main liver mass of the rabbit after ligation of its bile duct.

If the rate of the hypertrophy is approximately the same after local portal deprivation as after local ablation, this might mean either that the tissue deprived of portal blood is useless to the organism or that hypertrophy goes on irrespective of its activities. The pros and cons cannot be profitably discussed, but both alternatives entail the assumption that the liver is wholly a portal organ, finding its reason for being in the substances carried to it on the portal blood and in them only. The biliary activity of the atrophic tissue does not constitute evidence against such a view, even granting that the substances from which bile pigment is produced come to the liver normally on the portal blood alone—an assumption yet to be proved. The small hepatic mass which receives the entire portal blood after local diversion of the stream must be thought of as unable to cope with its functional opportunities for some time, so that much material for liver activity passes through into the general circulation and reaches the atrophic competing mass. Later, as the compensating tissue attains the size and functional power of the whole original liver, less of the portal material may be supposed to escape through it. Yet

¹⁶ von Meister, V., *Beitr. path. Anat. u. allg. Path.*, 1894, xv, 1.

¹⁷ Nasse, *Verhandl. deutsch. Ges. Chir.*, 1894, xxiii, 525.

that a portion does pass through the normal liver has been shown by Van Slyke¹⁸ for the amino-acids. It is perhaps a similar passage of material that enables a liver remnant wholly deprived of portal blood and advanced in atrophy to continue the formation of bile pigment, albeit in reduced quantity.

(c) *Food Deprivation.*—Though the main liver mass of our animals undoubtedly received relatively little of some at least of the substances upon which it normally acts, this need not have been the essential cause of its atrophy. Perhaps a special food is essential to liver maintenance. Whether this comes from a systemic or portal source, the tissue receiving the whole portal stream would have the best opportunity at it, and, possessing the superior powers of a growing, healthy tissue, would gradually increase its rival's deprivation. But were the source of the hypothetical food substance systemic, not portal, one would expect atrophy and hypertrophy to go on more slowly than is the actual case and perhaps not to reach completion. The question whether food substances can be distinguished from those utilized in function need not be entered upon.

Much of the foregoing incomplete analysis is only warranted in as far as it illustrates the vital importance for the liver tissue of a position on the portal stream. A situation there is not obligate, it is true. For the liver deprived of the direct portal stream by an Eck fistula still survives,—though perhaps only because it still receives the portal substances, at one remove, so to speak, by way of the systemic circulation.

Influence on Liver Development.

Toldt and Zuckerkandl¹⁹ demonstrated in 1876 that the normal human liver undergoes notable changes in shape during the period from birth to adult life. In some portions of the organ atrophy occurs, while in others there is hypertrophy. The atrophy seems to be identical with that after portal diversion, while, when it is complete, as not infrequently happens, there are left behind the same large corded ducts and blood vessels. Toldt and Zuckerkandl attribute the changes to pressure from the surrounding organs and adduce

¹⁸ Van Slyke, D. D., *Arch. Int. Med.*, 1917, xix, 56.

¹⁹ Toldt and Zuckerkandl, *Sitzungsber. k. Akad. Wissensch. Wien.*, 3te Abt., 1876, lxxii, 241.

reasons for their belief. We would go a step further and suggest that the transmitted pressure may produce its effect in some instances through local alterations in the portal stream.

Mall,²⁰ who confirms the findings of Toldt and Zuckerkandl, points out in another connection that the distribution of the portal blood to the hepatic parenchyma is in general remarkably even, as can be demonstrated by injection methods. No hepatic region is specially favored. In the light of our observations the necessity for this is clear. For any enduring local irregularity in the portal flow will result in a shift of parenchyma. One of the commonest shifts observed by Toldt and Zuckerkandl entailed a complete atrophy of the left lobe. Herringham²¹ found ten such cases in 3,000 autopsies. A reason for this is not far to seek, nor for the rarity of atrophy of the right lobe. In man the right branch of the portal vein is extremely short and thick, breaking up almost at once into many lesser vessels; whereas the left branch courses for a long distance through the parenchyma as a single slender trunk, much exposed to transmitted pressure.

Bearing on Liver Lesions.

Pathologists have long recognized that liver destruction frequently induces a local compensatory hypertrophy. From our observations it is evident that there exists, conversely, a type of destruction dependent upon compensatory hypertrophy. The knowledge should aid in an understanding of certain chronic liver lesions. The advanced local atrophy sometimes occurring in livers containing an echinococcus cyst, a gumma, slow growing tumor, or other limited process may well be the result of pressure upon portal radicles. In such instances there is present elsewhere in the organ an abundance of parenchyma capable of compensatory proliferation. The opportunities for marked changes are far less favorable in the atrophic cirrhosis of Laennec. The irregular stenosis and occlusion of portal branches which characterize the disease fail to lead to a complete atrophy of large liver portions because the parenchyma which under ordinary circumstances would proliferate in compensation is prevented from so

²⁰ Mall, F. P., *Am. J. Anat.*, 1906, v, 227.

²¹ Herringham, W. P., *St. Bartholomew's Hosp. Rep.* 1905, 1906, xli, 15.

doing by a confining connective tissue. However, numerous small areas of partial hypertrophy and atrophy may and do exist.²² In syphilitic livers with sharply localized scarring no such impediment is present; and we would suggest that local portal obstruction is a prime cause for the extreme atrophy and hypertrophy which in such cases frequently lead to great hepatic distortion. According to Sternberg²³ a whole lobe of the syphilitic *hepar lobatum* may be reduced to a connective tissue appendage.

The disappearance of large masses of liver tissue without the least connective tissue replacement may take place in the very old. According to MacCallum²⁴ whole layers of parenchyma may disappear, . . . "on the surface of the organ blood-vessels, bile-ducts, and the fibrous skeleton of the liver lie exposed." Not infrequently the atrophy is one of deprivation, and identical in its essentials with that resulting from local portal occlusion. improvement

SUMMARY.

The occlusion of portal branches to a part of the liver of the rabbit leads to a progressive and ultimately complete atrophy of the parenchyma in the region deprived of portal blood, and to hypertrophy of the rest of the hepatic tissue which receives such blood in excess. Three-fourths of the liver may thus be reduced to a fibrous tag within 2 months, while the remaining fourth attains the bulk of the entire original organ. The atrophy is simple, unaccompanied by obvious degenerative changes or by any connective tissue replacement. More important, it is conditional in nature, failing to progress when the bile duct from the proliferating tissue is ligated and its hypertrophy checked in this way.

There are indications in the literature that an atrophy conditional on hypertrophy, such as is here described, occurs in man after local portal occlusion. And some experiments in our laboratory, not yet completed, show definitely its occurrence in the dog. The changes take place slowly in the canine liver. After 3 months the tissue de-

²² MacCallum, W. G., *J. Am. Med. Assn.*, 1904, xliii, 649.

²³ Sternberg, C., in Aschoff, L., *Pathologische Anatomie*, Jena, 1911, ii, 855.

²⁴ MacCallum, W. G., *Text-book of pathology*, Philadelphia and New York, 2nd edition, 1914, 60.

prived of portal blood has diminished to about one-third of its original bulk. The conditional character of the atrophy is proven by its failure to occur to any similar degree in the absence of a compensating parenchyma, as when the portal stream is diverted from the whole liver by way of an Eck fistula.

Is the atrophy functional? If so, its completeness would indicate that the liver has no essential activity—none on which its maintenance depends—that it is not intimately connected with substances derived from organs drained by the portal system. Observations on the rate of hypertrophy after local diversion of the portal stream and on the character of the bile secreted by the atrophic tissue may be taken to favor such a view. The hypertrophy is nearly, perhaps

CORRECTION.

On page 630, Vol. xxxi, No. 5, May 1, 1920, line 13, for *Not infrequently*
Not impossibly.

shape of the normal liver that have been loosely attributed heretofore to pressure from the surrounding organs. It also has some interest in connection with pathological changes. Liver hypertrophy dependent on a preceding destruction has long been known to pathologists. Now a type of destruction dependent on compensatory hypertrophy must also be reckoned with. The occurrence of changes of the latter character will explain certain of the lesions observed in diseases that involve a disturbance of the portal flow to portions of the liver substance.

EXPLANATION OF PLATES.

PLATE 67.

FIGS. 1 and 2. Rabbit 7, Table I. Hepatic atrophy and hypertrophy, respectively, 12 days after diversion of the portal stream from the main liver. Three lobules of the main liver are barely equal in size to one of the hypertrophic lobe mass. The cells in atrophy are smaller and appear crowded together. Hematoxylin and eosin.

PLATE 68.

FIG. 3. Rabbit 9, Table I. Pale spots on the surface of a main liver 12 days after the ligation of its portal trunk. To the right is seen the corresponding hypertrophic lobe mass.

FIG. 4. Rabbit 27, Table I; 118 days. The effect of portal collaterals. The main liver is in complete atrophy save for a small, button-like area of healthy looking parenchyma, which receives at its center a portal venule from the lesser curvature of the stomach. Fig. 9 is a photograph of the entire specimen, and Fig. 10 shows the microscopic findings in the main liver.

PLATE 69.

FIG. 5. Rabbit 21, Table I; 55 days. Advanced atrophy of the main liver. The parenchyma is greatly diminished in amount, and the surviving lobules are extremely irregular. Bile ducts and blood vessels are prominent and numerous, and the intralobular capillaries are much widened in this special instance, but the lobules themselves are uninvaded by connective tissue which, however, is definitely increased. The increase was present prior to operation, as the liver fragment taken at the time shows. The dark rounded masses here and there are Kupffer cells distended with pigment. Hematoxylin and eosin.

FIG. 6. Rabbit 25, Table I; 68 days. Another instance of advanced atrophy of the main liver, but with pigmented Kupffer cells in unusual abundance. The peculiar appearance of the liver cells is due to the fixative. The great number of bile ducts relative to parenchyma should be noted, as also the characteristic absence of any increase in connective tissue, save for a slight thickening about the ducts, that was present prior to operation. Hematoxylin and eosin.

PLATE 70.

FIG. 7. Rabbit 24, Table I; 65 days. A late stage of parenchymal disappearance. Two small islands of liver cords with characteristic capillaries can be discerned. Many ovoid, pigmented Kupffer cells are present here and there. Methylene blue and eosin.

FIG. 8. A highly magnified parenchymal island from the same specimen. Near it are individual liver cords and cells isolated by the enveloping connective tissue. To the right and left lie Kupffer cells distended with pigment. Methylene blue and eosin.

PLATE 71.

FIG. 9. Rabbit 27, Table I; 118 days. Practically complete atrophy of the main liver, with compensatory hypertrophy of the lobe mass. In Fig. 4 a nearer view of the main liver is given.

FIG. 10. Condition of the main liver in the same rabbit. Save for an occasional cell, which cannot be discerned in the picture, the parenchyma is entirely gone. There remain arteries, veins, and bile ducts in a slight matrix of connective tissue, with some aggregations of round cells. Eosin and methylene blue.

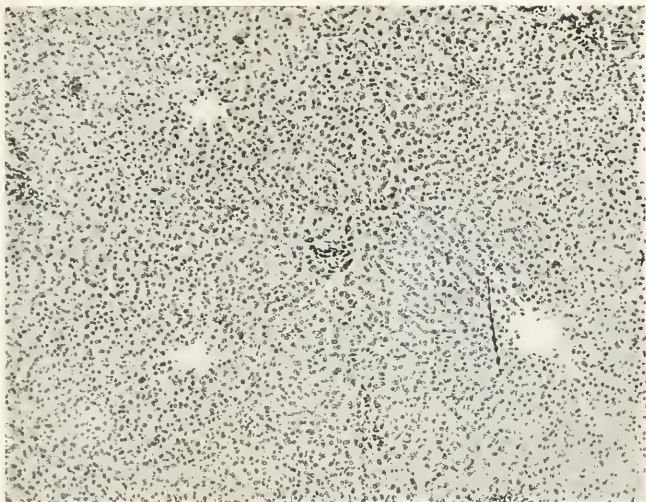


FIG. 1.



FIG. 2

(Rous and Larimore: Portal blood and liver maintenance.)

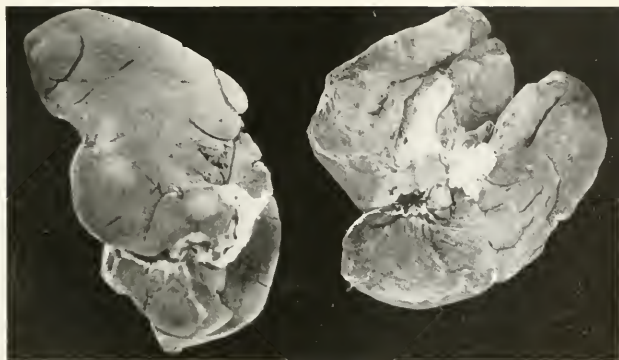


FIG. 3.

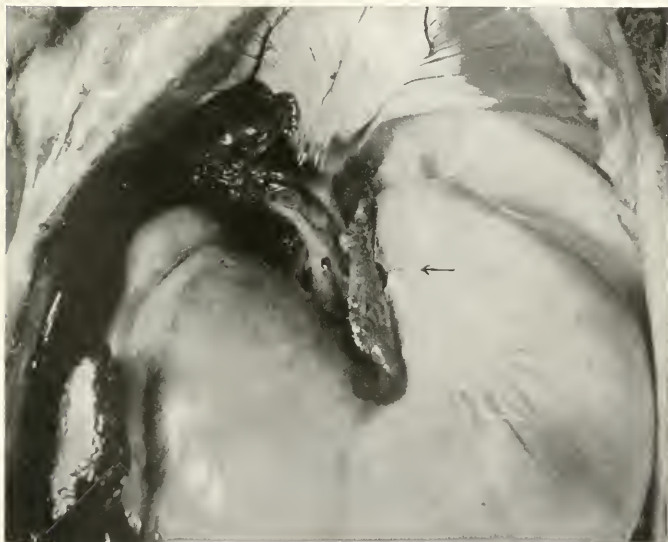


FIG. 4.

(Rous and Larimore: Portal blood and liver maintenance.)



FIG. 5

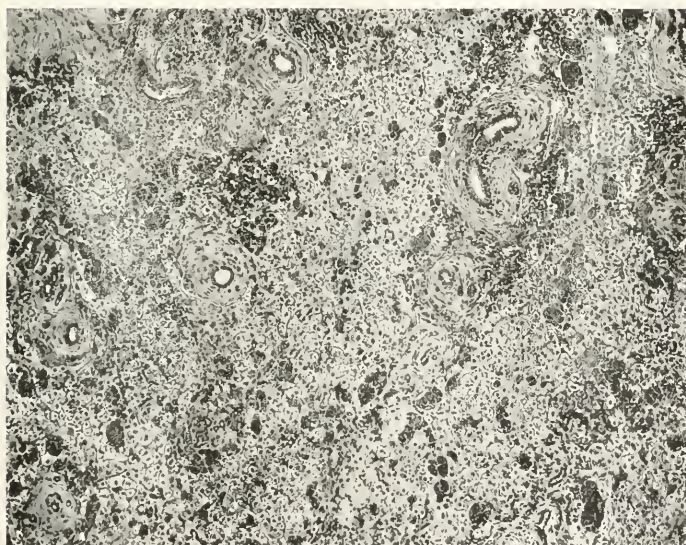


FIG. 6.

(Rous and Larimore: Portal blood and liver maintenance.)

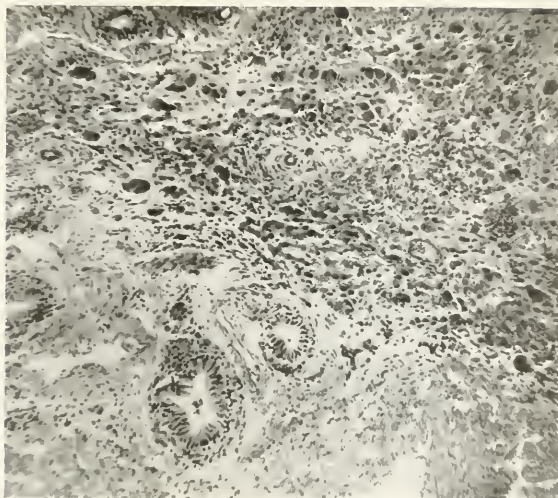


FIG. 7.

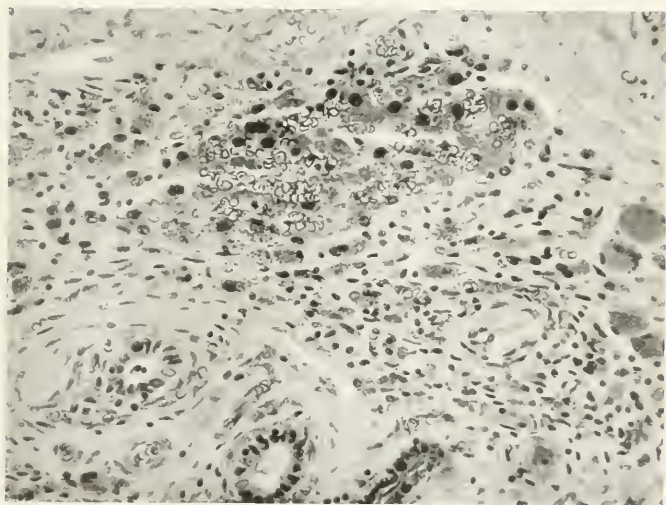


FIG. 8.

(Rous and Larimore: Portal blood and liver maintenance.)

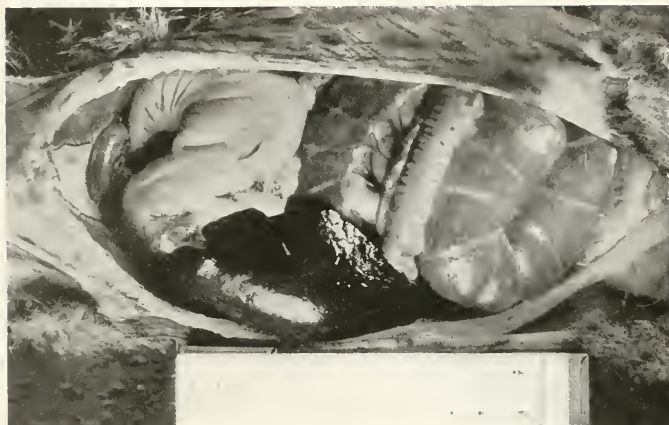


FIG. 9.

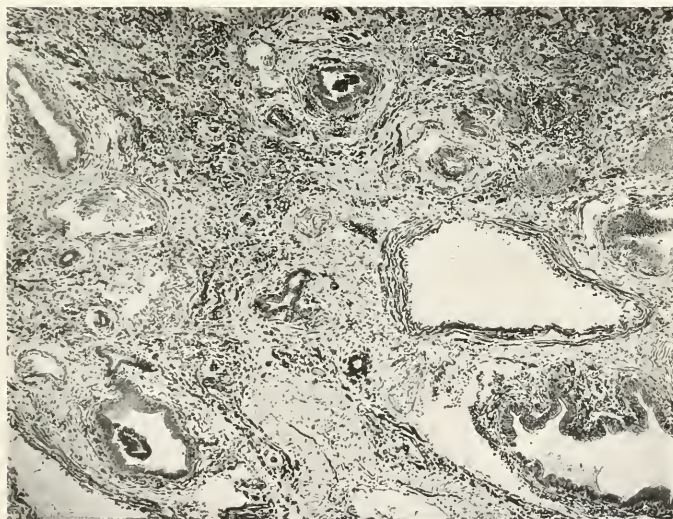


FIG. 10.

(Rous and Larimore: Portal blood and liver maintenance.)

EPIDEMIOLOGY OF BLACKHEAD IN TURKEYS UNDER APPROXIMATELY NATURAL CONDITIONS.

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The field experiments to be described in this paper are continuations of those made during the warm season of 1916 and published the following year.¹ The results of this earlier work indicated that the exposure of young incubator turkeys to older birds from infected flocks on infected ground produced disease within 2 weeks; exposure to older infected birds on fresh territory also produced disease, but after a somewhat longer period, and exposure to manifestly diseased young stock did not yield positive results. The last mentioned outcome might lead to various inferences, such as the elimination of unripe stages during active disease, the existence of an intermediate host, the need for a period of incubation in the soil, and so on.

The work of the years 1917, 1918, and 1919 was designed to repeat that of 1916, while furnishing ample material for the prosecution of other lines of work. However, as will be shown, the exposure of young turkeys to infected stock yielded cases of disease at irregular and uncertain intervals, so that intensive work on more specific problems had to be postponed until a method of producing blackhead with certainty and within a definite period of time could be worked out.

The experiments were conducted as heretofore with turkeys raised in the incubator and brooder and subsequently confined in outdoor enclosures of limited extent, varying in area from about 300 to 10,000 square feet. Each enclosure contained a small house in which the turkeys were kept at night. No attempt was made to cover these enclosures so as to exclude rodents, sparrows, and migrating birds.

¹ Smith, T., *J. Exp. Med.*, 1917, xxv, 405.

For it was considered of importance to determine if possible whether carriers, other than turkeys, exist. All experiments unless otherwise stated were conducted with turkeys raised in the incubator and brooder.

The term "blackhead" which has found its way into common use as referring to the disease due to the invasion of the walls of ceca and liver with a specific protozoan parasite, *Amoeba meleagridis*, is used in these pages for convenience's sake.

Experiments of 1917.

Three separate experiments were made in three outdoor enclosures. One was designed to test the infectiousness of young diseased turkeys, the other that of poultry from large flocks. The third contained controls. The turkeys used were hatched in three lots on dates beginning May 23 and ending June 16.

Experiment 1.—To test the infectiousness of young diseased turkeys, the latter were obtained from a nearby farm. Of a lot of eleven hatched by a hen and a turkey, three had died and the diagnosis had been confirmed on one by autopsy. Aug. 20. Four of the remainder, 5 to 6 weeks old, were introduced into the experimental enclosure containing four healthy turkeys. Aug. 25. One infected turkey died of blackhead. Aug. 26. A second died of the same disease. Aug. 29. The third died of coccidiosis. Blackhead lesions were not detected. Aug. 30. The fourth died of blackhead complicated with coccidiosis. The incubator turkeys were thus exposed for 10 days to both blackhead and coccidiosis in four living birds, and after the death of the latter to whatever soil infection resulted, until Nov. 9, a period of more than 2 months. No deaths occurred and no illness was observed. Nov. 9. One of the four was killed and found free from any lesions. The droppings of these birds had been examined in the course of the experiment and at the close the droppings of all were sedimented and examined for coccidia and ova but none were found. It should be stated that in the infected birds there were found in addition to the coccidia, specimens of *Heterakis papillosa*.

Experiment 2.—Aug. 20. To test the effect of exposure to chickens, four White Leghorns from four different flocks, three from New Jersey and one from Massachusetts, were placed in an outdoor enclosure and four turkeys placed with them. Turkey 175 was hatched on May 23, Nos. 178 and 185 on May 29, and No. 190 on June 16. No. 175 was first noticed to be sick on Sept. 28, 39 days after the beginning of the experiment. It was chloroformed and characteristic lesions of blackhead were found in ceca and liver. One individual of *Heterakis papillosa* was found in one cecum. The other three turkeys remained well. No. 185 was killed on Nov. 8, over 2½ months after the beginning of the exposure, and was

found free from any lesions or scars indicative of an attack. Four individuals of *Heterakis papillosa* were present. The two remaining turkeys were not killed but the feces were collected, washed, and sedimented. Neither ova nor coccidia were found.

Experiment 3.—Control enclosure. Eight incubator turkeys left after taking out those used in Experiments 1 and 2 were kept in an outdoor enclosure during the entire summer (Nos. 176, 179, 180, 182, 184, and 186 to 188). Aug. 25. No. 176 died. The feet of this turkey had been deformed and it was finally unable to walk. No blackhead lesions were present. Oocysts of coccidia were found in the ceca. No. 186 became sick on Sept. 14 and was killed on Sept. 21. Blackhead lesions were present in ceca and liver. No. 179 was killed Nov. 6 and No. 180 on Nov. 7, to determine if the birds had passed through a mild attack. No lesions or scars were present in either bird. Worms, ova, and coccidia were absent. The feces of all but No. 176 had been examined during the last week in Aug. and coccidia found in Nos. 184 and 187.

This group of experiments is significant from several points of view. It shows that blackhead may appear in an isolated group of turkeys and suggests that birds other than turkeys or hens may be carriers of the parasite. The disease appeared first in the control group on September 14 and 2 weeks later in the group with the chickens. It is highly probable that the same outside agencies brought the disease to both groups and that the chickens were not responsible. Singularly, the turkeys associated with three fatal cases of blackhead and one of coccidiosis remained healthy during the entire season, thus confirming earlier work that young diseased birds do not transmit blackhead. In accordance with this fact is the low morbidity in Experiments 2 and 3. Although each group was exposed to a case of blackhead occurring among its own numbers in September, yet no further illness occurred during the remainder of the season.

Experiment 4.—Towards the close of 1917 some individuals of the three groups (Experiments 1, 2, and 3) were brought together in the enclosure of Experiment 1 with a male, No. 125, which had been raised in the incubator in 1916 and exposed to disease during the summer of that year. This bird was therefore 1½ years old. A second group was formed on the grounds recently occupied in Experiment 3 as a control. Table I summarizes the condition when the groups were broken up on June 14, 1918. One in each group had been killed in Jan. and found normal. All in the control group were well. In the other group one died of blackhead on May 15 and two were ill about the same time, but recovered. They may have

passed through an attack of blackhead. One had been injured by the male and this may have accounted for the illness in one case. Both were still alive at the beginning of 1920. Several interpretations may be put on the outcome. Blackhead may have been transmitted by the old infected male (see Experiment 6), or by some individuals from Experiments 1, 2, and 3 which had become carriers, or by some outside agency.

TABLE I.

Exposed in enclosure of Experiment 1, Nov. 9, 1917, to Male No. 125, until June 14, 1918.

No. of turkey and group to which it had belonged.	Subsequent history.
181 (1)	June 14, 1918. Well.
189 (1)	Jan. 28, 1918. Killed. Normal. <i>Heterakis papillosa</i> in ceca.
184 (3)	May 23, 1918. Sick. Recovered.
188 (3)	" 15, 1918. Died. Blackhead.
178 (2)	" 20, 1918. Sick. Recovered.
Controls (in Enclosure 3).	
182 (3)	June 14, 1918. Well.
187 (3)	" 14, 1918. "
190 (2)	" 14, 1918. "
183 (1)	Jan. 30, 1918. Killed. Normal. <i>Heterakis papillosa</i> in ceca.

Experiments of 1918.

Experiment 5.—This experiment was performed to test the relative infectiousness of older turkeys which had been exposed in the preceding year.

The flock to which young turkeys were to be exposed consisted of one from Experiment 1, two from Experiment 2, and three from Experiment 3 of 1917. With this flock the male referred to in the preceding experiment, No. 125, remained for a short time. They had occupied the same enclosure since the preceding year. The infectious character of the environment was demonstrated by the fatal case of blackhead of May 15, referred to in Experiment 4.

To imitate the usual conditions, one of these turkeys was made to incubate ten eggs. Six hatched on May 22. One young was killed in the nest, leaving five. One died on June 13, probably of general weakness. No infection could be demonstrated. On this day the remaining four were allowed to run with the older turkeys. All died of blackhead, one on July 22, one on Aug. 11, one on Aug. 13, and the last one on Sept. 8. After the death of the young stock, three additional young incubator turkeys were exposed on Sept. 17. Two of these contracted the disease on Oct. 2 and Nov. 12, respectively. The third, killed on

Nov. 25, 1919, showed some suspicious smooth areas in the ceca, but no definite lesions were found.

Two turkeys were introduced into this enclosure as late as Nov. 29. One of these contracted blackhead in Jan., 1919. The other remained well.

To test the susceptibility of chickens, four, hatched in an incubator and running only with incubator turkeys, were placed in this enclosure on Sept. 3. They

TABLE II.

Turkey No.	Source.	Exposure.	Result.
254	Hatched by No. 181 on May 22.	Exposed to mother turkey in coop until June 13, then to the old flock.	July 22. Died of blackhead, after 39 days.
255	Hatched by No. 181 on May 22.	" "	Aug. 11. Died of blackhead, after 59 days.
256	Hatched by No. 181 on May 22.	" "	Aug. 13. Died of blackhead, after 61 days.
257	Hatched by No. 181 on May 22.	" "	Sept. 8. Died of blackhead, after 87 days.
242	Hatched by No. 181 on May 22.	" "	June 13. Died, general weakness.
	Hatched by No. 181 on May 22.		Killed by accident in the nest.
264	Hatched in incubator.	Exposed on Sept. 17.	Oct. 5. Chloroformed. Affected with blackhead.
263	Hatched in incubator.	" " " 17.	Nov. 25, 1919. Killed. Normal except for a few possible scars in ceca.
262	Hatched in incubator.	" " " 17.	Nov. 19. Chloroformed. Affected with blackhead.
265	Hatched in incubator.	" " Nov. 29.	Jan. 21, 1919. Chloroformed. Affected with blackhead.
248	Hatched in incubator.	" " " 29.	Remained well.
Four chickens.	Hatched in incubator.	" " Sept. 3.	All remained well.

remained well and were killed, two 65 days and two 80 days after the beginning of the exposure. No lesions were found. Table II summarizes the results of this experiment.

Probably the most significant feature of this experiment is the fact that sooner or later nearly all the exposed died, but the period between the beginning of exposure and actual disease was very variable.

Experiment 6.—June 14. Four incubator turkeys were penned on new ground with the old male, No. 125, referred to in Experiments 4 and 5. July 9. Four additional young turkeys were placed in the same enclosure. Aug. 23. The old male was killed and found normal. Aug. 28. Experiment closed. None of the exposed showed signs of blackhead.

Experiment 7.—In this experiment young turkeys were penned with chickens from two sources. From each source a chicken had been found affected with blackhead. The first lot of chickens was penned with three healthy turkeys, beginning July 5. Dec. 20. Two of the turkeys were killed and found normal. The third was reserved.

Sept. 18. The second lot of chickens was penned with two turkeys. After an exposure of 69 days, one turkey was killed and found normal. The other was reserved for breeding.

TABLE III.

Lot No.	Date of hatching.	No. in each hatch.	Beginning of outdoor life.	Cases of blackhead.	No. running together at the end of.
	1918		1918		
1	Apr. 24	10	May 9	None.	June, 48.
2	May 12	25	" 27	Nov. 27. (One.)	July, 39.
4	" 26	24	June 3	Dec. 20. (")	Aug., 37.
5	" 28	(a) 10 (b) 8	" 1 " 3	June 22, Sept. 21, Nov. 6, 12, Dec. 14. (Five.)	Sept., 31. Oct., 30.
6	June 13	15	" 15	Oct. 26. (One.)	Nov., 15.

Experiment 8.—A study was made of the epidemiology of a large flock not exposed experimentally to disease. In the spring of 1918 the possibility that the eggs might be infertile led to the incubation of a relatively large number of eggs. The resulting hatch yielded a larger number of poults than could be taken care of adequately. The history of this group is therefore of interest from a practical standpoint. The various broods which went into the large flock are given in Table III.

The ground covered by the enclosure and the surrounding acres had not been used for poultry for several years and had been ploughed and planted in the spring. A small flock of incubator chickens was permitted to mingle with the turkeys to some extent. The entire group of turkeys was herded on an adjoining tract of several acres under supervision from time to time and then returned to the enclosure. When the vegetation in the latter had been destroyed the fence was moved along to cover fresher ground. The maximum number together at any time was 52, in the middle of June. Withdrawals and deaths from blackhead, diseases other than blackhead, and accidents, and the killing of eleven for food in Nov., gradually reduced the total number to fifteen at the end of Nov.

Among the miscellaneous early causes of death were crowding and chilling in early June, leading to twenty deaths. Four died following unthriftiness and emaciation, two of impaction of the small intestine, due to eating coarse food, three with congestion of lungs, one with inflammation of the ceca, and one with impaction of the kidneys with urates. These losses, thirty-one in all, occurred up to June 15, and might have been largely averted if the accommodations had been adequate to prevent overcrowding.

About the middle of June withdrawals began to be made for experimental purposes. Miscellaneous causes of death continued operative, although on a much smaller scale. One died with ceca inflamed, one from congestion of lungs, and one from an undefined cause, late in June. Two were lost from undefined causes and one from unthriftiness, in July. Among the infectious diseases, aspergillosis appeared early in July. Two birds died of this disease; two were chloroformed and the foci discovered at autopsy.

Blackhead appeared first in June. In all, eight cases were discovered. The distribution in time was quite irregular, as shown in Table III. Thus one case occurred in June, one in Sept., one in Oct., three in Nov., and two in Dec. The remaining twelve were killed in Dec. and no lesions found in any.

Ten from this same lot, which had been removed to other enclosures during the season, were killed late in Nov. and early in Dec., and all found free from traces of blackhead lesions.

Experiments of 1919.

Experiment 9.—The fortuitous appearance of blackhead in enclosures protected in every way against the disease, except as it might be brought in by birds on the wing or small rodents, is well illustrated by the history of several flocks gradually merged into one larger flock during the summer of 1919.

The territory occupied during the season was an unused horse paddock enclosed by a high iron fence and not occupied by poultry for many years. The ground had been ploughed early in the spring and oats and grass sown. May 21. The first group of eight turkeys was moved to a brooder in the paddock when 9 days old. June 3. The brooder yard was enlarged to give more room. June 12. The brooder was replaced by a larger house. In the meantime one turkey had succumbed to blackhead on June 9. Others died on June 22, 29, July 2, 9, and 12. Two survived. To determine the infectious character of this flock and the soil, four additional young turkeys were introduced on June 30 (of which one died of softening of the bones soon afterward), three on Aug. 18; four on Sept. 5; and nineteen from another flock also on Sept. 5. In this last group, one had succumbed to blackhead on Aug. 13.

Sept. 5. The entire paddock was opened to the flock which now comprised thirty-one birds. Oct. 16. Twelve were taken out for a special feeding experiment. Oct. 20. One of the remaining nineteen died of blackhead. Nov. 17. Nine were taken out for another feeding test. Of the nine remaining, seven were

killed late in Dec. and all found free from lesions or scars of blackhead lesions. The ceca of all contained adult *Heterakis*, one bird carrying as many as twenty-nine specimens. The two left from the entire flock were penned with the older infected flock for breeding purposes. One of these died of acute blackhead on Jan. 30, 1920. Most of the data are brought together in Table IV.

The points of interest in the history of this flock are several. The unexpected, severe outbreak in June among birds still in the brooder resembles closely the occurrences so frequent upon farms where turkeys are raised. Thereafter only one other case occurred, making a mor-

TABLE IV.

Lot No.	Date of hatching.	No. of individuals.	Introduced into enclosure.	Cases of blackhead.	Remarks.
	1919		1919		
1	May 12	8	May 21	One died on June 9, 22, 29, July 2, 9, 12.	June 3. Yard enlarged.
1	" 12	4	June 30	(One dies of softening of bones.)	
2	July 2	3	Aug. 18	One dies on Oct. 20.	Sept. 5. Entire paddock opened up to the thirty-one turkeys running together on Sept. 5.
2	" 2	4	Sept. 5	None died. (One had died in this group on Aug. 13.)	
3	May 27	19	" 5		

talities of seven among thirty-eight birds. The other feature of interest is the absence of immunity among the survivors, although all had been exposed at one time or another to a case. Of six fed subsequently with infectious material, all died. One of the original lot, of which two survived out of eight, died over 6 months later of the acute disease, after having been penned with older infected turkeys for over a month.

Experiment 10.—June 18. In another enclosure placed on land ploughed up and seeded to oats and grass in the spring, a flock of twenty-three incubator turkeys, hatched on June 10, was placed. June 24. Six were taken out for experimental uses, leaving seventeen. July 13. A turkey died, possibly of coccidiosis,

as oocysts were abundant. Blackhead appeared in the middle of Aug. and cases occurred well into Nov., as shown in Table V.

Although only three out of ten died, yet the symptoms of the rest and the presence of suspicious smooth, often deeply pigmented areas in one or both ceca and of whitish scars or foci in the liver make the diagnosis of blackhead fairly certain. Possibly the first case might be eliminated. This interpretation would push the beginning of the epidemic into Sept.

After counting out the dead and recovered cases, there were left six which had not shown signs of disease. They were killed late in Nov. The organs were free from lesions or scars. *Heterakis* was abundant in all instances. The extent

TABLE V.

Turkey No.	Beginning of illness.	Result.	Further observations.
	1919		
332	Aug. 19	Recovered.	Nov. 22. Killed. Normal.
354	Sept. 9	"	" 25. " Some scars in liver and smooth areas in ceca.
355	" 13	"	Reserved for breeding.
356	" 28	"	Jan. 21, 1920. Killed. Scars in liver and smooth and dark pigmented areas in ceca.
357	" 30	"	Nov. 24. Killed. Some whitish spots on liver and pigmented areas in one cecum.
358	" 30	Oct. 24. Died.	Blackhead.
359	Oct. 4	" 24. "	"
363	" 21	Nov. 13. "	"
364	Nov. 1	Recovered.	Nov. 22. Killed. Whitish foci in liver and pigmented areas in ceca.
365	" 6	"	Reserved.

of the disease may perhaps be accounted for by the fact that this flock was allowed to run over an adjoining part of the land on pleasant days, in the care of an attendant who drove them back into the smaller enclosure after 1 or 2 hours.

Experiment 11.—In this experiment a spontaneous outbreak among turkeys penned with incubator chickens occurred. The enclosure was placed on land which had been ploughed up and sown to grass and oats in the spring.

There were nineteen chickens in the flock. July 28. Two young turkeys were penned with them. Both became ill in 23 and 26 days respectively. One was chloroformed and the diagnosis of blackhead confirmed. Young stages of *Heterakis* were present. The other recovered and was killed in Nov. The liver showed healed foci and there were found a constriction of one cecum due to scar tissue and smooth areas in both ceca. Sept. 5. Three additional turkeys were placed in this group. All three contracted blackhead, one after 31 and the others after

34 days. One died. Mature *Heterakis* were found in the ceca. The others recovered. Both were killed in Jan., 1920, and in each there were changes indicative of healed blackhead lesions. The chickens remained clinically well. Eight were killed early in Oct. and two late in Nov. No lesions were detected. *Heterakis papillosa* was present in the ceca.

This group is of interest from the fact that all five turkeys placed with the chickens contracted blackhead. The relatively short period of incubation may have been due to the comparatively late exposure, since all experiments have pointed to an accumulation of disease-producing factors with the advance of the summer. The bearing of the chickens on the incidence of blackhead is not clear. It seems as if they may have picked up the virus with *Heterakis* and cultivated it with the latter in the ceca.

Experiment 12.—This experiment was designed to test the infectiousness of an enclosure recently occupied by a group of older turkeys as compared with the infectiousness of the same group on fresh soil.

(a) *Exposure to Older Turkeys on New Ground.*—The flock consisted of seven hens and a male, hatched during 1917 and 1918. June 2. They were cleansed and all soil was washed from the feet and they were then placed on grounds not heretofore used for poultry. The coops and nests were thoroughly scrubbed and cleansed before they were moved to the new grounds. June 3. Six young turkeys 7 days old were transferred in a brooder to the same new grounds and allowed to run out during warm weather. During cold or rainy days they were kept in the large coops occupied by the older turkeys at night to increase opportunities for infection. June 16. They were permitted to run freely with the older birds. In the meantime one had been smothered by the others. The remaining five did well up to Aug. 6, 64 days after the beginning of the exposure, when one became sick. One became sick on Aug. 7, one on Aug. 10, and two others on Aug. 15. One was killed on Aug. 20 and the diagnosis of blackhead confirmed. Some young and nearly adult *Heterakis* were found in the ceca, but no coccidia. The four remaining turkeys gradually recovered and all were well by the end of Aug. Evidence that they had passed through an attack was furnished when they were killed, two late in Dec. and two towards the end of Jan., 1920. Constrictions, obliterations of the longitudinal folds and pigmentation in the ceca, and scars or grayish foci in the liver were present.

In the group of twenty-two turkeys from which the above were taken one case occurred on Aug. 4. On Sept. 5 this group was merged with another group and one other case occurred in the combined flocks during the remainder of the season.

Aug. 23. The experiment was continued by placing three fresh turkeys into the enclosure with the older turkeys. The concentration of infectious material which had been going on was shown by the rapidity with which this new lot be-

came affected. One was ill on Sept. 4 and died on Sept. 12. The second was ill on Sept. 6 and it was killed on Sept. 9 and found diseased. Individuals of *Heterakis* were present in an immature stage in both cases. The third bird showed signs of illness on Nov. 17, but recovered. When killed on Jan. 22, 1920, the only indication of former disease was a large white healed focus in the liver. *Heterakis papillosa* was present.

(b) *Exposure on Grounds Vacated by Older Turkeys.*—The grounds occupied by the older flock during the winter were cleared, as stated under (a), on June 2, and remained vacant for 22 days. June 24. Six turkeys 2 weeks old were placed in it. 30 days later a young turkey showed signs of drooping and died of blackhead 15 days later. Three others contracted the disease, two on July 29 and one on Aug. 5. In all, both ceca were diseased. In two a few coccidia were found and in two larval nematodes. The fifth became sick on Sept. 27 and recovered. When killed late in Jan., 1920, there were a number of large scars in the liver and a smooth patch in each cecum. Nov. 2. The last of the six, an exceptionally vigorous bird, showed signs of disease and died 3 days later of blackhead.

Meanwhile, the loss of the birds was partly made good by introducing four young healthy turkeys on Aug. 18, when they were 47 days old. These also contracted blackhead. One died on Sept. 25, one on Oct. 20, and one on Oct. 21. The fourth showed signs of disease on Oct. 4, but recovered. It was killed on Jan. 22, 1920. The liver showed a few whitish foci and each cecum a smooth area on the mucosa.

In this experiment, the intensity of the infectious agent seems to have been nearly equal in the two enclosures. Disease appeared 30 days after the beginning of the exposure, in the vacated enclosure, and 64 days in the new enclosure containing the older birds. There is evidence that the infection became, if anything, more rather than less concentrated as the season progressed. This was indicated by the shorter time elapsing between exposure and disease.

The fact that 100 per cent of the exposed birds contracted the disease neutralizes the possible error involved in using birds from flocks in which spontaneous disease occurred. The percentage of such cases in the stock flocks was low and the disease first made its appearance some time after it had begun in the experimental enclosures.

One flock which consisted originally of twenty-three incubator turkeys, hatched on July 2, deserves mention here since blackhead did not appear in the enclosure during the greater part of the summer season. They were placed on a plot of ground on July 4, and allowed to run in a small yard on July 8 and in a larger enclosure on July 23. The flock was gradually reduced by withdrawals, four on

August 7, four on August 18, and three on August 23. Some died of non-infectious troubles. Seven were still present on September 5. These were later used in other experiments. They had thus been on the soil 59 days without any symptoms of blackhead.

During October a mild form of chicken-pox appeared in all but one of the groups of turkeys. It was first noticed among the turkeys in Experiment 9, and later in Experiments 12, *b*, 10, and 12, *a*. The mode of introduction remains unknown. This epizootic had no appreciable influence on the health of the birds.

SUMMARY.

The foregoing experiments in outdoor, unprotected enclosures demonstrate the difficulties surrounding the rearing of turkeys. These are discussed from another view-point² and to avoid repetition only a few outlying facts should be considered here.

The occasional presence of coccidia, the presence of *Heterakis papillosa* in the ceca, the occurrence of cases of aspergillosis and of chicken-pox in incubator-bred birds which did not come in contact with other domesticated birds, except in a few cases with incubator-bred chickens, show clearly that turkeys are picking up from the ground material deposited by other birds. The agent of blackhead must come from the same sources.

The field experiments show a steadily increasing concentration of the infection from 1917 to 1919, even though the ground had been ploughed and seeded before use. As a result, the various groups of turkeys became infected to a greater degree. The growth in the intensity of the disease may be in part ascribed to an accumulation on the soil of infectious agents during any given season after they had been introduced, but it is hardly acceptable as an explanation from season to season, when the soil was either virgin, as regards poultry yards, or ploughed deep and seeded before use. A more rational hypothesis is the gradual attraction of birds in larger numbers and greater variety on account of the food supply in the turkey enclosures and the more intensive cultivation of the land surrounding the laboratory and animal buildings since the beginning of the experiments in 1917.

² Graybill, H. W., and Smith, T., *J. Exp. Med.*, 1920, xxxi, 647.

The intensity of the outbreaks due to the confining of young turkeys with birds over a year old which had been infected during the preceding year, or on grounds previously occupied by them, was in all instances much greater than in the spontaneous outbreaks. The cases amounted to nearly 100 per cent of the exposed. On the other hand, the number of cases in the control flocks varied and was very low in some groups. It could have been kept down if the sick birds had been promptly removed and not permitted to recover on the same ground. However, the object of the experiment was not to suppress the disease, but to see to what extent it would develop.

It is self-evident that the results obtained apply strictly only to that part of the country where the experiments were made. We have at present no means of knowing whether the sources of infection would become more numerous and concentrated with a higher mean annual temperature, or the reverse. Only by using incubator turkeys exclusively for such tests and eliminating the older turkeys and domesticated birds as carriers, can the miscellaneous, at present not controllable sources of the agents of this disease in different localities and the chances of successful rearing be determined.

PRODUCTION OF FATAL BLACKHEAD IN TURKEYS BY FEEDING EMBRYONATED EGGS OF HETERAKIS PAPILLOSA.

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Since the protozoan agent of blackhead was described and its relation to the lesions of the ceca and liver defined by one of the authors¹ in 1895, very little concerning the mode of transmission of the agent or the conditions favoring its invasion and multiplication has come to light. In 1895 Moore² fed the liver and ceca of three diseased turkeys to two healthy ones. The days of feeding were Nov. 28, Dec. 3, and Jan. 3. On Jan. 11, 44 days after the first feeding and 8 days after the last feeding, one of the turkeys died of blackhead. In another experiment beginning Nov. 28, Moore penned four healthy turkeys with two diseased ones. During the night for a period of about 2 weeks the feces of the sick ones were collected and fed, mixed with the feed, to the well ones on the following day. One of the two sick turkeys died in 3 days and the other survived. Of the four which received the discharges and were also exposed during the day to the sick ones and their environment, three became diseased. Two died in 22 and 27 days respectively; the third bird, well when killed 46 days after the beginning of the experiment, showed extensive lesions of blackhead.

In Sept., 1913, one of the authors³ fed two incubator turkeys, hatched early in June of the same year and reared on virgin soil, the chopped up diseased ceca of two turkeys 2 to 3 months old. Both remained clinically well up to Dec. 24 when one was stolen. An autopsy on the other 3 days later showed it to be normal.

New Experiments in Feeding Feces.

During the years 1918 and 1919 certain attempts to induce blackhead by feeding feces of older turkeys which had successfully passed through exposure to disease in the preceding year were made. These

¹ Smith, T., *U. S. Dept. Agric., Bureau Animal Industry, Bull. 8*, 1895, 7.

² Moore, V. A., *U. S. Dept. Agric., Bureau Animal Industry, Circular 5*, 1896, 1.

³ Smith, T., *J. Med. Research*, 1915, xxxiii, 243.

attempts, although leading uniformly to negative results, are detailed below because of their bearing on subsequent more successful trials. It should be stated here that cohabitation in the same enclosure with the turkeys furnishing the feces produced blackhead in a certain number of young turkeys.

Experiment 1.—June 10, 1918. A male bird, 2 years old, was confined in an isolation unit after all soil had been washed from its feet. The feces were collected and fed to four young turkeys confined in a cage in the same unit. The turkeys were 30 days old. The feces were mixed with the food and fed on June 11, 12, 13, and 14. The experiment was closed on July 9 and the turkeys were released. At no time during a period of 28 days did they show symptoms of blackhead. These turkeys were used in other experiments during the season but none of them developed blackhead. One died on July 22 of a disease of the bones, resulting in all probability from confinement in the cage. The others were killed at the close of the year, one on Dec. 10 and the others on Dec. 20. The organs were uniformly normal.

Experiment 2.—Three turkeys, hatched on May 12, 1918, were fed feces of two old turkeys under the following conditions: The feces were collected on Sept. 2 and 3. A portion was fed on Sept. 2 with the food, morning, noon, and night. The remainder was placed in Petri dishes at room temperature until fed. The later feedings took place on Sept. 6, 9, and 13.

The young turkeys had been running with some chickens in a special enclosure and were kept in it except on the dates of the feeding. The feeding was done in a cage. They remained well and when two were killed late in Dec. no lesions were found. The older turkeys from which the feces were obtained belonged to an enclosure in which young had contracted blackhead. It will be noted that in this trial an incubation of the feces at room temperature for 3, 6, and 10 days respectively failed to make the test positive.

Experiment 3.—The feces of adult turkeys were fed to chickens.

In view of Milks⁴ observations on the occurrence of blackhead in young chickens, it was believed that very young chickens would be suitable for feeding experiments, and such an experiment was undertaken in the spring of 1919, before young turkeys were available. This experiment was conducted in a brooder and was begun on Apr. 4, when the chicks were 8 days old. Six chicks were used, two of each of the following breeds: Rhode Island Red, White Leghorn, and Barred Plymouth Rock. The feces were collected as fresh as possible from the coops occupied by the old flock kept for breeding purposes. It consisted of eight birds, and in the main contained the same ones that were in it the previous year. Feces having different physical characters were collected. Those not fed fresh were kept in Petri dishes at room temperature. In all, from Apr. 4

⁴ Milks, H. J., *Louisiana Agric. Exp. Station, Bull.* 108, 1908, 1.

to 17, feces were fed on 12 days. They ranged in age, counting from the date of collection, from less than 1 to 5 days. The chicks at no time showed symptoms of blackhead. One showed lameness due in all probability to confinement.

The manner of feeding the feces was then modified to reproduce more nearly natural conditions. On Apr. 17 a galvanized iron tray was placed in the outer compartment of the brooder and filled with sterilized soil. Feces were placed from time to time on the soil and the feed was always placed there. The soil was kept moist. Fresh and 1 day old feces were placed on the tray on Apr. 17 and on Apr. 18, 19, 20, 22, 23, 24, and 25 fresh feces were placed there.

Aside from lameness the chicks showed no other symptoms. Three of them were chloroformed on May 2, and three on May 6, 28 and 32 days respectively after the beginning of the experiment, and all were found free from blackhead.

After it had been found that by placing young turkeys with the old flock, subsequent to removal to new soil, they contracted blackhead, it was decided to undertake still another experiment feeding the feces of the old flock to young turkeys.

Experiment 4.—The turkeys of the old infected flock were removed by twos and placed in an indoor unit to facilitate collecting feces of all alike. The feces not fed fresh were kept in Petri dishes at room temperature. Beginning on Aug. 7, four young turkeys 36 days old, two white and two bronze, were fed in a cage. The feeding was carried out daily, with the exception of Sunday, to Aug. 19. After the feeding they were still kept indoors, but in a pen to enable them to be more active. The feces from eight older birds were used. When fed, they were either fresh or up to 8 days old. On Sept. 5 the young turkeys were removed to an outdoor enclosure. 23 days after the last feeding the experiment was closed. None had shown signs of disease in the meantime. Owing to a shortage of incubator turkeys these were used again in later experiments.

Feeding Experiments with Embryonated Eggs of Heterakis papillosa.

In experiments conducted in 1914,³ 1916,⁵ and since that time, relating to the mode of transmission of the disease, it appeared that young diseased turkeys which did not recover were incapable of transmitting blackhead to healthy ones penned with them. In addition to this, it became evident that soil recently occupied by old turkeys was infectious to young ones.⁶ These observations along with negative results in feeding feces suggested the existence of some

³ Smith, T., *J. Exp. Med.*, 1917, xxv, 405.

⁶ Smith, T., and Graybill, H. W., *J. Exp. Med.*, 1920, xxxi, 633.

additional factor or factors in the transmission of blackhead. Previous experience and experiments gave no direct clue as to the nature of such a factor. There were a number of hypotheses which might fit the conditions observed or at least not be contradicted by them. The protozoan parasite invading the walls of the ceca and the liver evidently multiplied very rapidly when once started. It was also evident that this multiplication came to an abrupt stop and that the parasites in the tissues perished rapidly owing to an acquired immunity of the host or some other factor operating against the invading parasite at the height of the invasion. It was assumed that this parasite might be aided by some lesion or injury of the mucosa of the ceca to enter the tissues and multiply there, since the invasion in itself did not seem to be a normal part of the life cycle of the parasite. There was no evidence of the formation of any resistant stages in the tissues or of the normal discharge of the parasite outward, as, for instance, in coccidiosis. If the parasite was not to be regarded as wholly aberrant, whatever normal cycle there was seemed to belong to the lumina of the ceca. In harmony with this hypothesis a nematode parasite, *Heterakis papillosa*, occurring in the ceca of turkeys, chickens, and some other birds, was brought into the experiment as the possible associated factor.

Experiment 5.—This experiment consisted in feeding to young turkeys embryonated eggs of *Heterakis papillosa* and feces from adult turkeys. Worms were collected from the ceca of healthy chickens, killed for this purpose, by washing and sedimenting the contents and picking out the worms. These were cut up in a small quantity of normal salt solution to liberate the ova. The suspension was kept in Petri dishes at room temperature and distilled water added from time to time to make up for evaporation.

After the cultures had been kept for 17 days, examination showed that the ova contained living embryos. On Sept. 11 they were added to the feed of two turkeys which had been used in the preceding experiment. To ensure the complete ingestion of the mixture the turkeys were placed in a cage. On the same and the 2 following days feces from two older turkeys, collected on Sept. 10 and 11 were fed to the same birds. They were then placed with two other turkeys, also from the preceding experiment, which were to act as controls, in an outdoor enclosure.

Both infected turkeys became sick on Sept. 26; *i.e.*, 15 days after the feeding of ova. One died of blackhead after 3 days, the other after 6 days. The two control birds remained well and 43 days after the beginning of the experiment they were used in Experiment 7.

Experiment 6.—In this experiment the two factors, embryonated eggs of *Heterakis papillosa* and feces of adult turkeys, were kept distinct.

The young turkeys used in this experiment were from a group consisting of portions of three different broods of incubator turkeys, numbering in all about thirty individuals.⁶ They had been running together for some time in a large enclosure. In some unknown way the parasite of blackhead had been introduced into this enclosure and six out of eight turkeys in one brood died of this disease during June and the first few days of July before the broods were mingled. Following these cases one turkey died of blackhead on Aug. 13 and another on Oct. 20. *Heterakis papillosa* was known to be present from some of the autopsies made. The latter was the only case that appeared in the remainder of the flock after the birds chosen for this experiment had been removed. Of the turkeys used Nos. 308, 309, and 312 were hatched on May 12, 1919, Nos. 360, 361, and 362 were hatched on May 27, and Nos. 345, 346, 350, 351, 352, and 353 on July 2. The turkeys were separated into four groups on Oct. 16: Nos. 309, 312, and 352 (controls) were fed nothing. Nos. 346, 350, and 360 received embryonated eggs plus turkey feces. Nos. 345, 353, and 361 received embryonated eggs only. Nos. 308, 351, and 362 received turkey feces only.

The *Heterakis* eggs used in this experiment were obtained from worms collected from the ceca of four chickens on Oct. 1, and were prepared for culture and incubated as in Experiment 5. The feces were obtained from two older turkeys of the infected flock, the same ones that were used in the preceding experiment, and were less than 48 hours old when fed.

The three feedings of ova and feces, of ova, and of feces, respectively, were conducted in separate enclosures and the three groups of turkeys were brought together after the feeding in a new enclosure with the controls, care being taken to remove all traces of soil from the feet by washing them thoroughly before the groups were finally assembled.

The result of the experiment is briefly told. Of the group which was fed eggs plus feces, Nos. 346 and 360 showed symptoms on Oct. 28, and No. 350 on Oct. 29; *i.e.*, in 12, 12, and 13 days respectively. They were killed and autopsied a few days later and the diagnosis was confirmed by the lesions in ceca and liver. Of the group fed ova only, Nos. 345 and 361 showed symptoms on Oct. 27, and No. 353 on Oct. 29; *i.e.*, in 11, 11, and 13 days respectively. Several days later they were killed and the autopsy showed marked lesions of ceca and liver.

The three that were fed feces only showed no symptoms of disease and were killed and autopsied, two on Nov. 10, and one on Nov. 11; *i.e.*, about 2 weeks after all those fed eggs had become sick. No lesions were found in these birds. A careful search for *Heterakis* in the cecal contents showed the presence of one mature male in No. 308, eight mature males in No. 362, and four females, two males, and one larval nematode in No. 351.

The three control birds showed no symptoms of disease and were killed on Nov. 10 and 11. One of these (No. 352) had lesions of blackhead; the others were entirely normal. In the ceca of No. 309, two mature males and one female, and in

No. 312, one female *Heterakis* were found. In the affected control both ceca showed many elevated indurations up to 0.5 cm. in diameter. The largest had a central superficial slough or scab. In the liver only a few small whitish foci, 1 to 2 mm. in diameter, and one focus of congestion were found. In fresh preparations from the ceca a few *Heterakis* eggs and one larval worm were found. In the washed and sedimented feces two mature and two immature females of *Heterakis* and many larval worms with attenuated posterior extremity and a bulbous esophagus were found. The mouth was without buccal capsule and provided with four papillae.

The lesions of this case suggest that the disease may have been produced by something that occurred after the beginning of the experiment. This bird may have ingested ova from the adult worms in the other birds or perhaps embryonated eggs passing through the intestines of the others soon after they had been fed. In any case, the causes bringing about disease in this instance will probably be cleared up in future experiments when the external sources of the two factors, *Amaba meleagridis* and *Heterakis papillosa*, shall have been more definitely located.

Experiment 7.—This test may be regarded in a way as supplementary to Experiment 5. The two controls there surviving were fed 43 days after the beginning of Experiment 5 with embryonated eggs of *Heterakis papillosa*. The worms were collected from four chickens and the ova incubated as heretofore for 16 days, when they were fed mixed with the daily ration. The feeding was done on Oct. 24. One turkey became ill on Nov. 4, the other on Nov. 6; i.e., 11 and 13 days after feeding. They were chloroformed and the diagnosis was confirmed by finding lesions of ceca and liver.

Experiment 8.—Owing to the lateness of the season, only one additional test was undertaken. This consisted in feeding the contents of the ceca of turkeys affected with blackhead which had died or had been chloroformed and the feces of older turkeys, both incubated for 15 days, but free from *Heterakis* eggs.

The contents of the ceca of four diseased turkeys were suspended in normal salt solution and passed through a No. 40 wire mesh. The strained suspension was placed in Petri dishes in shallow layers. The incubation began on Nov. 3. Careful examination of the fluid under a low power failed to show the presence of ova.

Nov. 3. The feces of older healthy turkeys were collected, suspended in water, and passed successively through a tea strainer and wire screens of Nos. 40, 60, 100, and 200 mesh respectively. The final sediment was washed twice in normal salt solution and distributed into four Petri dishes. Nov. 14. The incubated dishes were carefully examined under a low power. Two *Trichosoma* ova but no others were detected.

Nov. 18. Nine turkeys, taken from the group from which those in Experiment 6 came, were selected. Three were reserved as controls, three fed cultures from diseased turkeys, and three cultures from older turkeys. After the feeding the three groups were brought together in one outdoor enclosure. No symptoms of disease appeared, and the birds were killed and autopsied as shown in Table I.

It will be noted that the turkeys were killed 21 and 29 days after the experiment had been begun. In the collection of the worms the contents of the ceca were washed and sedimented. Lesions were uniformly absent.

TABLE I.

Turkey No.	Date killed.	No. of <i>Heterakis</i> individuals.
	1919	
310	Dec. 9	1 adult.
366	" 17	8 adults.
367	" 17	14 "
368	" 9	15 "
369	" 17	15 "
370	" 17	10 "
371	" 17	19 "
372	" 17	8 "
373	" 9	6 " and 2 young males.

DISCUSSION AND SUMMARY.

In four experiments, three with young incubator turkeys and one with young incubator chickens, in which the feces of old turkeys from an infectious flock, kept at room temperature up to 5, 8, and 10 days, were fed, no infection resulted.

In an experiment in which two of four young incubator turkeys used in one of the above experiments were fed embryonated eggs of *Heterakis papillosa* and feces of turkeys from an infectious flock both contracted blackhead. Two controls remained well. Later they were fed embryonated eggs of *Heterakis papillosa* and both contracted blackhead.

In another experiment three incubator turkeys received embryonated eggs plus turkey feces from an infectious flock. All contracted blackhead. Three received embryonated eggs alone; all contracted blackhead. Three received turkey feces only; none contracted blackhead. Three controls received nothing; one showed blackhead lesions at the autopsy.

In a final experiment three turkeys were fed cultures of feces from the ceca of diseased turkeys, three were fed cultures of feces of old turkeys from an infected flock, and three controls were fed nothing. None contracted blackhead. The cultures of feces were prepared

precisely as were the earlier ones containing *Heterakis* eggs but without the latter.

From these experiments it becomes evident that blackhead may be produced in healthy incubator-raised turkeys, reared in the open in an environment where blackhead occurs, but out of direct contact with old turkeys and other poultry, by feeding cultures of embryonated eggs of *Heterakis papillosa*, prepared by cutting up the worms in isotonic salt solution and incubating the suspension at room temperature.

These very definite and clear-cut results outweigh any objections which may be raised against the use of turkeys which had been in earlier experiments and which came through such experiments without any signs of disease, or which came from control flocks in which spontaneous cases had occurred. The short time elapsing between feeding embryonated eggs and the first signs of disease made these experiments unusually impressive. It should be stated, furthermore, that from a precise individual record of all turkeys it was possible to select birds from control flocks in which the infection had either not appeared or was very low. All but two turkeys in flocks serving as sources of this material were killed at the close of the year. None at any time had shown symptoms of disease, and no scars or other abnormalities of ceca and liver were found. Furthermore, all other control birds and those in field experiments, with the exception of two reserved for breeding, were likewise killed. As a result of these autopsies, it was determined that of all birds in which symptoms of disease had not been recorded during life, none showed abnormalities or scars at autopsy. The protozoan factor in blackhead was probably disseminated when the first spontaneous cases occurred in the stock, unless it was present and made invasive by incubation in the cultures fed. This latter theory seems at present not acceptable because of the wholly negative outcome of Experiment 8.

The production of acute blackhead by feeding embryonated eggs to turkeys in whose ceca adults of *Heterakis papillosa* are already present seems incomprehensible at first thought. A tentative explanation to be offered is that the worms when invading the ceca in large numbers break down the resistance of the bird which is able to protect itself against a few. This may account for the very irregular

occurrence of cases in contact with older recovered birds on infected grounds. The rôle of *Heterakis* as a preliminary agent may also account for the continuing high mortality in turkeys in which the disease has been operating for so many generations to eliminate the most susceptible. It now seems highly probable that the turkey has become relatively resistant to the invasion of the protozoan parasite acting alone and that such invasion may require other agencies. Whether *Heterakis papillosa* is the only, or at any rate, the chief accessory agent or whether there are others, living or inert, which when ingested by the turkey assist in preparing the way for the destructive invasion of the walls of the ceca and the liver by *Amæba meleagridis* is a question now open to solution by experimentation.

The relation of common poultry to outbreaks of blackhead may be accounted for, at least in part, by the fact that they are hosts of *Heterakis papillosa*. How frequently they also carry *Amæba meleagridis* remains to be determined.

Since earlier communications have contained certain practical suggestions on the rearing of turkeys and the prevention of blackhead, it is not out of place here to point out that the additional information presented in this article simply emphasizes the suggestions already made. Turkeys should be raised in the incubator and brooder and kept away from older turkeys and poultry. The shelters should be moved from time to time to prevent a too concentrated infection of the soil with *Heterakis* ova. Inasmuch as the factors producing blackhead may be deposited by certain still undetermined birds on the wing, disease may be looked for at any time during the warm season. It is not, however, very readily transmitted, and in the experiments described elsewhere⁶ the mortality from spontaneous blackhead was low. The flock should be looked over as frequently as possible, and whenever a turkey begins to droop, it should be isolated and killed if the drooping continues over several days. If such turkeys are allowed to recover, they should not be returned to the young flock but kept with older, presumably infected birds. Such birds are entirely satisfactory as a source of eggs, since there is no evidence that the latter transmit the infection.

STUDIES ON EXPERIMENTAL PNEUMONIA.

V. ACTIVE IMMUNITY AGAINST EXPERIMENTAL PNEUMOCOCCUS PNEUMONIA IN MONKEYS FOLLOWING VACCINATION WITH LIVING CULTURES OF PNEUMOCOCCUS.

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In a preceding paper¹ it was shown that the subcutaneous injection of killed pneumococci in doses comparable to those employed in man will not protect monkeys against pneumococcus pneumonia of homologous or heterologous type. The aim of the present study has been to determine the prophylactic value of a vaccine composed of living pneumococci.

The conviction, based on considerable experimental evidence, has long been held by immunologists that a living virus stimulates a more effective resistance to infection than a killed virus.

Metchnikoff and Besredka,² in their studies on typhoid vaccination in the chimpanzee, found that very little protection was conferred by vaccination with dead typhoid bacilli, whereas animals vaccinated with small amounts of living cultures were efficiently protected. Haffkine's³ method of vaccination against cholera consisted in the injection of 0.2 to 0.05 cc. of living culture of the cholera vibrio, first an attenuated culture being used and 5 days later a more virulent one. As early as 1893 Haffkine and his coworkers vaccinated 40,000 people in India by this method and obtained, on the whole, encouraging results. Kolle and Otto,⁴ after experiments upon monkeys and other animals, concluded that dead plague bacilli were much inferior to attenuated living cultures for prophylactic vaccination.

¹ Cecil, R. L., and Blake, F. G., *J. Exp. Med.*, 1920, xxxi, 519.

² Metchnikoff, E., and Besredka, A., *Ann. Inst. Pasteur*, 1911, xxv, 193; 1913, xxvii, 597.

³ Haffkine, W. M., *Bull. Inst. Pasteur*, 1906, iv, 825.

⁴ Kolle, W., and Otto, R., *Deutsch. med. Woch.*, 1903, xxix, 493; *Z. Hyg. u. Infektionskrankh.*, 1903, xlv, 507.

Strong⁵ made a thorough study of the various methods of plague vaccination and concluded that the most efficient method was immunization with attenuated living cultures. He showed that when carefully done this method can be employed in human beings.

The investigations referred to above indicate that for the diseases of bacillary origin at least, living vaccines are in many ways preferable to dead cultures.

The experiments included in this study may be divided into two groups: (1) vaccination with a living virulent culture of *Pneumococcus* Type I, and (2) vaccination with a living avirulent culture of *Pneumococcus* Type I.

EXPERIMENTAL.

Two species of monkeys were used in this investigation, *Cebus capucinus* and *Macacus syrichtus*.

Method of Vaccination.—The inoculations were administered subcutaneously in the abdominal wall. In the experiments with the virulent culture the dose of vaccine was in all instances 0.001 cc. of an 18 hour broth culture diluted to 1 cc. with normal salt solution. This amount of culture contained approximately 400,000 pneumococci. In vaccinating with the avirulent strain a dose of 1 to 2 cc. of undiluted broth culture was employed.

Reactions.—The local reaction to living pneumococcus vaccine was very mild in the monkeys which were studied, consisting of merely a slight induration, free from redness and tenderness. The constitutional reaction depended, on the one hand, upon the dose and virulence of the culture, and, on the other hand, upon the individual resistance of the monkey. The latter is a factor of considerable variation. As the experiments will show, the constitutional reactions varied widely in severity, from no reaction whatever to a fatal pneumococcus septicemia.

The resistance of the vaccinated monkeys to experimental pneumonia was tested 2 or 3 weeks after vaccination by the intratracheal injection of virulent pneumococci, as described in Paper I.⁶

⁵ Strong, R. P., *J. Med. Research*, 1908, xiii, 325.

⁶ Blake, F. G., and Cecil, R. L., *J. Exp. Med.*, 1920, xxxi, 403.

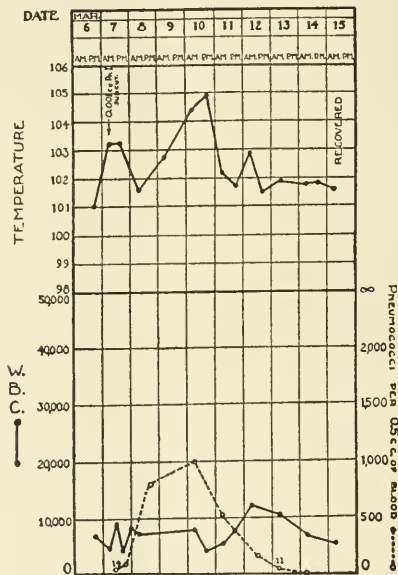
Vaccination with a Living Virulent Culture of Pneumococcus.

The strain of pneumococcus used as a vaccine in this group of experiments was a highly virulent *Pneumococcus* Type I which killed a mouse regularly in doses of 0.0000001 cc. of broth culture. The same strain was used for the intratracheal injections, and when injected into the trachea of monkeys in doses of 0.000001 cc. of broth culture would produce in practically every instance a classic lobar pneumonia.

Reaction of Monkeys to Subcutaneous Injection of Living Virulent Pneumococci.—The first monkey vaccinated with a living virulent pneumococcus was studied in considerable detail. The protocol therefore will be reported in full.

Experiment 1.—Monkey 7. *Cebus capucinus*, female; weight, 1,430 gm. Mar. 7, 1919, 10.30 a.m. Well and active. Subcutaneous injection of 0.001 cc. of 18 hour broth culture of *Pneumococcus* Type I. 1.30 p.m. Well and active. Blood culture: 0.5 cc. in broth, *Pneumococcus* Type I; 0.5 cc. in agar plate, 14 colonies of *Pneumococcus* Type I. 4.30 p.m. Appears well and active. Blood culture: 0.5 cc. in broth, *Pneumococcus* Type I; 0.5 cc. in agar plate, 84 colonies of *Pneumococcus* Type I. 7.30 p.m. Appears well and active. Mar. 8, 10.30 a.m. Appears well and active. Blood culture: 0.5 cc. in broth, *Pneumococcus* Type I; 0.5 cc. in agar plate, 800 colonies (approximately) of *Pneumococcus* Type I. Mar. 9. Appears well and active. Mar. 10, 9 a.m. Monkey quiet at times, restless at times; evidently becoming sick. 10.30 a.m. Blood culture: 0.5 cc. in broth, *Pneumococcus* Type I; 0.5 cc. in agar plate, 1,000 colonies (approximately) of *Pneumococcus* Type I. 4 p.m. Appears sick; offers little resistance to handling; respirations not increased. Blood culture: one loop on surface of blood agar plate, 18 colonies of *Pneumococcus* Type I. Mar. 11, 10.45 a.m. Appears better; more active. Blood culture: 0.1 cc. in broth, *Pneumococcus* Type I; 0.5 cc. in agar, 510 colonies of *Pneumococcus* Type I. 4.30 p.m. X-ray of chest. There is no evidence of consolidation in the chest. Mar. 12, 10 a.m. Appears well and active. Blood culture: 0.5 cc. in broth, *Pneumococcus* Type I; 0.5 cc. in agar plate, 151 colonies of *Pneumococcus* Type I. Mar. 13, 10.30 a.m. Appears well and active. Blood culture: 0.5 cc. in broth, *Pneumococcus* Type I; 0.5 cc. in agar plate, 11 colonies of *Pneumococcus* Type I. Mar. 14, 11 a.m. Well and active. Blood culture: 0.5 cc. in broth, no growth; 0.5 cc. in agar plate, no growth. Mar. 15. Well and active. Mar. 27. Well and active. Monkey bled (10 cc.). Serum tests with *Pneumococcus* Type 1: Agglutinins, 0. Protection, 0.0001 cc., survived; 0.00001 cc., survived; 0.000001 cc., survived $4\frac{1}{2}$ days. Control, 0.00001 cc., died in 48 hours; 0.000001 cc., died in 48 hours.

Text-fig. 1 shows the temperature, leucocyte, and blood culture curves following vaccination. There was a sharp rise of temperature in response to the injection, but the leucocytes were unaffected. The most striking feature of the chart is the blood culture curve. The pneumococci invaded the blood stream within 3 hours after vaccination,



TEXT-FIG. 1. Monkey 7. Reaction following subcutaneous inoculation of 0.001 cc. of broth culture of living virulent *Pneumococcus* Type I.

and by the end of 24 hours there were nearly 1,000 pneumococci per 0.5 cc. of blood. In spite of this septicemia, however, the monkey did not appear seriously ill at any time. The blood cleared up rapidly, the temperature dropped, and 5 days after vaccination the monkey appeared well and active. It is surprising that a heavy septicemia of this kind could have been associated with such mild clinical symptoms.

It is also noteworthy that 20 days after vaccination the serum of this monkey protected mice against 1,000 times the minimal lethal dose of *Pneumococcus* Type I.

Experiment 2.—Apr. 15, 1919. Three *Macacus syrichtus* monkeys (Nos. 46, 47, and 48) were inoculated with a living virulent *Pneumococcus* Type I, each receiving the same dose that Monkey 7 received—0.001 cc. of broth culture subcutaneously. Condensed protocols are shown in Table I and Text-fig. 2.

TABLE I.

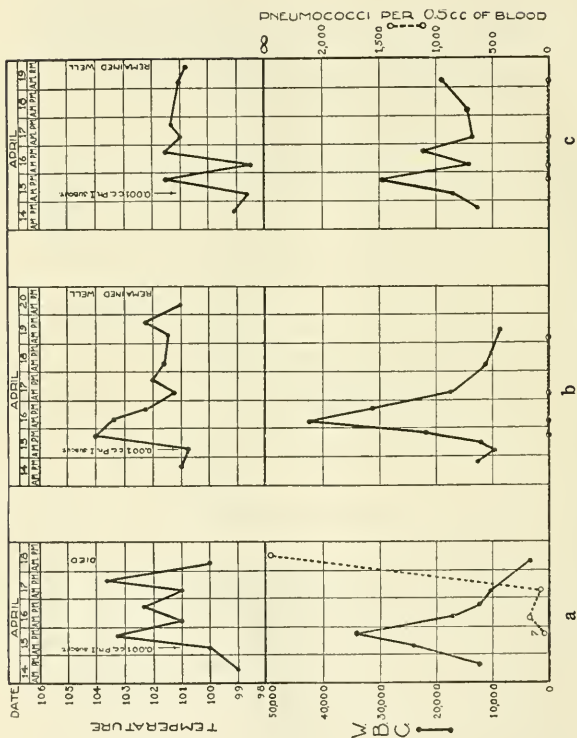
Effect of Subcutaneous Injection of Living Culture of Virulent Pneumococcus Type I.

Monkey No.	Weight.		Apr. 15. Broth culture of Pn. I subcuta- neously.	Apr. 29. Serum tests with Pn. I.			Result.	Autopsy.	Autopsy cultures. Heart's blood.
				Agglu- tinins.	Protection.	Control.			
	gm.	cc.							
46	2,550	0.001					D. 4 days.	Lungs normal.	Pn. I
47	2,710	0.001	1:1++	0.00001 cc. S.* 0.000001 cc. S. 0.0000001 cc. S.	0.00001 cc. D. 24 hrs. 0.000001 cc. D. 36 hrs. 0.0000001 cc. D. 36 hrs.		Remained well.		
48	3,590	0.001	0	0	0		Remained well.		

* S. indicates survived; D., died.

It will be observed that each of these three monkeys reacted differently to the vaccine. The smallest one (Monkey 46) showed a severe constitutional reaction with heavy septicemia, and died on the 4th day. Monkey 47 reacted with a sharp but temporary rise in temperature and leucocytes, but the blood remained sterile and the monkey at no time appeared ill. The largest monkey (No. 48) showed no reaction of any kind except a slight leucocytosis.

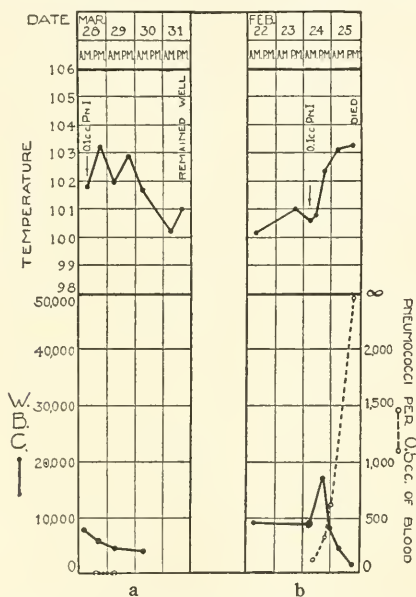
2 weeks after vaccination the two surviving monkeys were bled and their sera tested for agglutinins and protective bodies against *Pneumococcus* Type I. Monkey 47 showed both agglutinins and protective substances. Its serum protected mice against 100 times the minimal lethal dose of *Pneumococcus* Type I. Monkey 48, on the other hand,



TEXT-FIG. 2. *a*, *b*, and *c*. Reactions following subcutaneous inoculation of living virulent *Pneumococcus* Type I. (*a*) Monkey 46, (*b*) Monkey 47, (*c*) Monkey 48. Each of these animals received 0.001 cc. of living culture subcutaneously.

exhibited neither agglutinins nor protective bodies. These reactions indicate what a large factor individual variation may be in experiments of this kind.

Active Immunity Following Subcutaneous Injection of Living Virulent Pneumococci.—The immediate effect of subcutaneous injections of



TEXT-FIG. 3, *a* and *b*. Active immunity against *Pneumococcus* Type I following vaccination with living virulent *Pneumococcus* Type I. (*a*) Monkey 7; received 0.001 cc. of living culture subcutaneously. (*b*) Monkey 3; control.

living virulent pneumococci having been studied, the next step was to determine the degree of immunity conferred by this method of vaccination.

The object of the two following experiments was to test the vaccinated monkeys for active immunity against *Pneumococcus* Type I.

TABLE II.
Active Immunity Following Subcutaneous Injection of a Living Culture of Virulent Pneumococcus Type I.

Monkey No.	Weight, gm.	Mar. 7. Broth culture of Pn. I subcutaneously.	Mar. 11. X-ray.	Mar. 27. Serum tests with Pn. I.			Mar. 28. Broth culture of Pn. I intratracheally.	Result.	Autopsy.	Autopsy cultures.	
				Agglutins.	Protection.	Control.				Lung.	Heart's blood.
7	1,430	cc. 0.001	Negative.	0	0.0001 cc. S. 0.00001 cc. S. 0.000001 cc. S. 4½ days.	0.00001 cc. D. 48 hrs. 0.000001 cc. D. 48 hrs.	cc. 0.1	Remained well.			
3	1,595	0	" (Feb. 19).				0.1 (Feb. 24).	D. 2 days.	Lobar pneumonia; red stage.	Pn. I	Pn. I

The identical strain of *Pneumococcus* Type I was used for the intratracheal injections that had been used for the subcutaneous vaccinations.

Experiment 3.—Mar. 28, 1917. Monkey 7, 21 days after vaccination with living virulent *Pneumococcus* Type I, was injected intratracheally with 0.1 cc. of an 18 hour *Pneumococcus* Type I broth culture. No monkey of this species was available as an actual control at the time. The record of Monkey 3, however, is introduced to indicate what had been the invariable result when comparable doses of *Pneumococcus* Type I were injected intratracheally in normal unvaccinated *capucinus* monkeys. The temperature, leucocyte, and blood culture curves are charted in Text-fig. 3. The results are shown in Table II.

The vaccinated monkey (No. 7), though it received 100,000 times the minimal infecting dose, remained perfectly well, while Monkey 3 died with incipient lobar pneumonia and an overwhelming pneumococcus septicemia on the day following injection. Even the leucocyte count in Monkey 7 remained unchanged.

In other words, a very small quantity of living virulent pneumococcus culture (0.001 cc.) injected subcutaneously was sufficient to confer on the monkey a high degree of active immunity against the homologous strain.

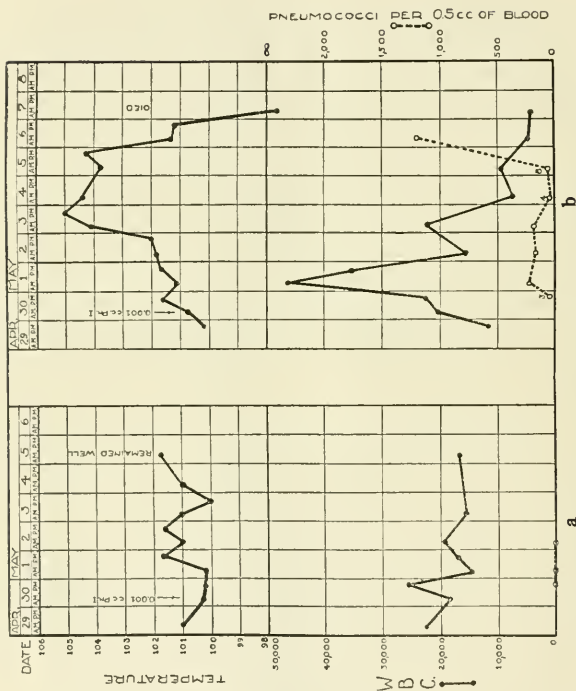
One of the *Macacus* monkeys (No. 48) which had been vaccinated with living virulent pneumococci (0.001 cc.) was next tested.⁷

Experiment 4.—Apr. 30, 1919. Monkey 48, vaccinated on Apr. 15 with 0.001 cc. of living *Pneumococcus* Type I, and Monkey 83, control, were each injected intratracheally with 0.001 cc. of *Pneumococcus* Type I broth culture.

The protocols (Table III and Text-fig. 4) show that the vaccinated monkey remained active and well. The control developed a typical lobar pneumonia and died on the 8th day with the usual pneumococcus septicemia. As in the previous experiment, the vaccinated monkey showed no rise in temperature and did not even react with a leucocytosis.

There was, however, a difference between the vaccinated monkey in Experiment 3 and the one in Experiment 4. The former developed a considerable amount of protective substance in the blood following vaccination; the latter showed none. Yet both were immune to *Pneumococcus* Type I pneumonia.

⁷ Monkey 47 will be considered later.



TEXT-FIG. 4, *a* and *b*. Active immunity against *Pneumococcus* Type I following vaccination with living virulent *Pneumococcus* Type I. (*a*) Monkey 48; received 0.001 cc. of living culture subcutaneously. (*b*) Monkey 83; control.

TABLE III.

Active Immunity Following Subcutaneous Injection of a Living Culture of Virulent Pneumococcus Type I.

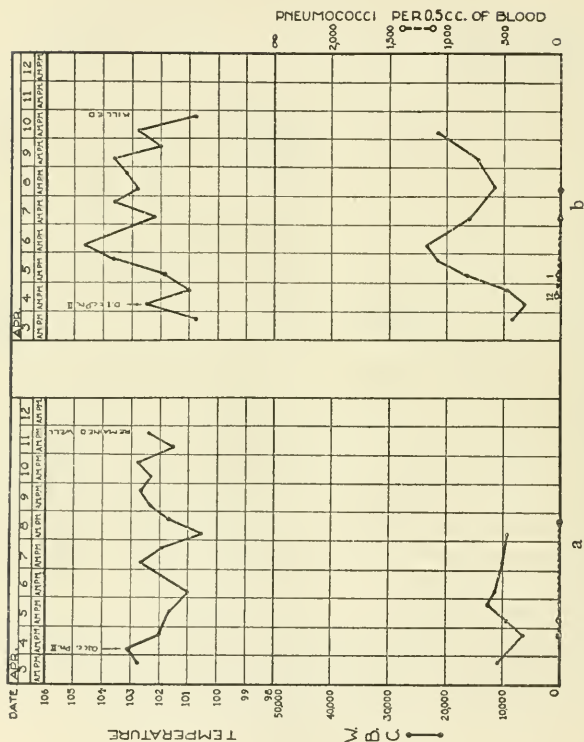
Monkey No.	Weight.	Apr. 15. Broth culture of Pn. I subcutaneously.	Apr. 30. Broth culture of Pn. I intratracheally.	May 2. X-ray.	Result.	Autopsy.	Autopsy cultures.	
							Lung.	Heart's blood.
	gm.	cc.	cc.					
48	3,590	0.001	0.001	Negative.	No signs of pneumonia. Remained well.			
83	2,470	0	0.001		Clinical pneumonia. D. 8th day.	Lobar pneumonia, R. U., R. M., R. L.*	Pn. I	Pn. I

*R. L., R. M., R. U., etc., indicate lobes of the lung. The cardiac lobe is included as part of the right lower lobe.

Cross-Immunity against Pneumococcus Type II Following Vaccination with Living Virulent Pneumococcus Type I.—The experiments just reported afford ample proof that vaccination with a living virulent *Pneumococcus* Type I stimulates a high degree of active immunity against the homologous type of pneumococcus. The next step was to determine whether vaccination with living *Pneumococcus* Type I afforded any cross-immunity against the other types of pneumonia. 1 week, therefore, after the test against *Pneumococcus* Type I, Monkey 7 was tested against a virulent strain of *Pneumococcus* Type II.

Experiment 5.—Apr. 4, 1919. Monkey 7, vaccinated against *Pneumococcus* Type I, and Monkey 25, control, were injected intratracheally with 0.1 cc. of broth culture of *Pneumococcus* Type II (Table IV, Text-fig. 5). Monkey 7 remained perfectly well, with no rise in temperature or leucocytes. Monkey 25, the control, developed lobar pneumonia with leucocytosis and positive blood culture. The control recovered by crisis on the 7th day.

According to this experiment vaccination with a living virulent *Pneumococcus* Type I had conferred not only immunity against the homologous type but a cross-immunity against *Pneumococcus* Type II as well. Furthermore, this immunity existed in spite of the absence of protective bodies against *Pneumococcus* Type II in the serum of the monkey.



TEXT-FIG. 5, *a* and *b*. Cross-immunity against Pneumococcus Type II following vaccination with living virulent Pneumococcus Type I. (*a*) Monkey 7; received 0.001 cc. of Pneumococcus Type I living culture subcutaneously. (*b*) Monkey 25; control.

TABLE IV.

Production of Cross-Immunity with a Living Culture of Virulent Pneumococcus Type I.

Monkey No.	Weight.	Mar. 31. Serum tests with Pa. II.		Apr. 4. Broth culture of Pa. II intra- tracheally.	Apr. 7. X-ray.	Result.	Autopsy.	Autopsy cultures.	
		Agglu- tina.	Protec- tion.					Lung.	Heart's blood.
	gm.			cc.					
7	1,430	0	0	0.1	Negative.	Remained well.			
25	740			0.1	Shadow, R. U.	Clinical pneumo- nia. Cri- sis on 7th day. Killed.	Lobar pneu- monia, R. U.; gray stage.	Sterile.	Sterile.

Experiment 6.—June 24, 1919. 8 weeks after the test against *Pneumococcus* Type I, Monkey 48, vaccinated against *Pneumococcus* Type I, and Monkey 91, control, were injected intratracheally with 0.1 cc. of broth culture of *Pneumococcus* Type II (Table V, Text-fig. 6).

Both monkeys developed Type II pneumonia and both recovered. In the vaccinated monkey, however, the disease ran a mild short course, and the blood was sterile throughout practically the entire course of the disease. The control monkey ran a long course (crisis on the 18th day) and showed a fairly heavy pneumococcus septicemia.

In the case, then, of Monkey 48 successful vaccination against Type I pneumonia did not give sufficient cross-immunity to protect against *Pneumococcus* Type II pneumonia, though it did apparently moderate the severity of the disease. The inconsistency between the results obtained in Experiments 5 and 6 may be explained in several ways. In Experiment 5, the test for cross-immunity against *Pneumococcus* Type II was made 1 week after the test against *Pneumococcus* Type I; in Experiment 6 the test for cross-immunity was carried out 8 weeks after testing for homologous immunity. The duration of pneumococcus immunity is not known, but a high degree of resistance lasts probably not more than a few months. Another ex-

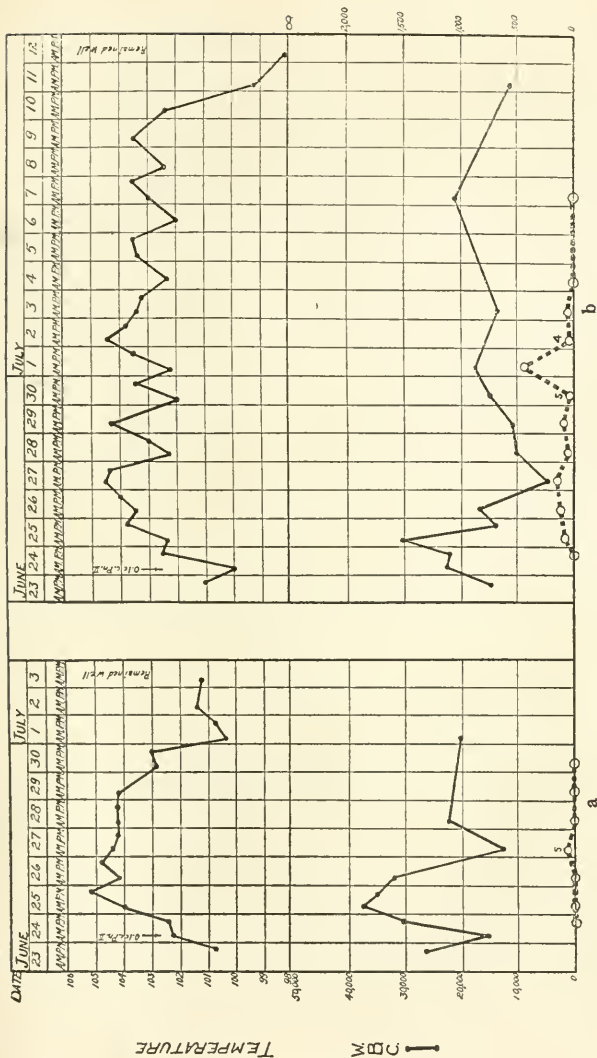
planation may be found in the much severer reaction exhibited by Monkey 7 following vaccination, with a consequent development of a higher grade of immunity. Still another and perhaps more rational explanation would interpret this difference in cross-immunity to individual variation in the monkeys.

TABLE V.

Experimental Pneumococcus Type II Pneumonia Following Vaccination with a Living Culture of Virulent Pneumococcus Type I.

Monkey No.	Weight.	Apr. 15. Broth culture of Pn. I subcutaneously.	Apr. 30.	June 23. Serum tests with Pn. II.			June 24. Broth culture of Pn. II intratracheally.	Result.
				Agglutina.	Protection.	Control.		
48	gm. 3,590	cc. 0.001	Resisted infection with Pn. I intratracheally.	0	0.000001 cc. S. 0.00001 cc. S. 0.0001 cc. D. 48 hrs. 0.001 cc. D. 48 hrs.	0.000001 cc. D. 48 hrs. 0.00001 cc. D. 20 hrs. 0.0001 cc. D. 18 hrs.	cc. 0.1	Clinical pneumonia. Recovery by crisis on 8th day.
91 (control).	4,002	0					0.1	Clinical pneumonia. Recovery on 18th day.

There is one more interesting feature to this experiment. It will be recalled that Monkey 48, though immune to Pneumococcus Type I pneumonia showed no protective substance in the blood. When, however, 8 weeks later a second protection test was carried out on the serum of this monkey, this time against Pneumococcus Type II, a definite though slight amount of protection was demonstrated. We offer no explanation of this phenomenon.



TEXT-FIG. 6, a and b. Pneumococcus Type II pneumonia following vaccination with living virulent Pneumococcus Type I. (a) Monkey 48; received 0.001 cc. of living culture of Pneumococcus Type I subcutaneously. (b) Monkey 91; control.

Cross-Immunity against Pneumococcus Type III Following Vaccination with Living Virulent Pneumococcus Type I.—The last two experiments (Nos. 5 and 6) gave some evidence of a cross-immunity against *Pneumococcus* Type II following vaccination with living virulent Type I pneumococci. It was decided, therefore, to determine whether a similar cross-protection existed against *Pneumococcus* Type III.

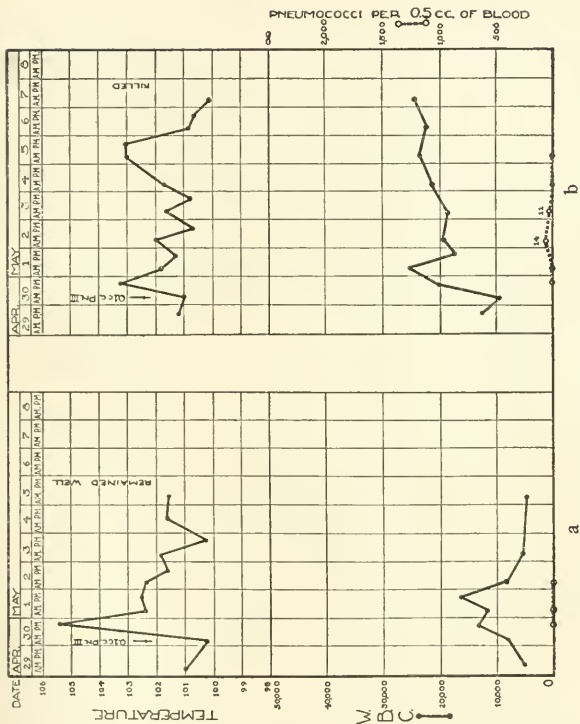
TABLE VI.

Production of Cross-Immunity with a Living Culture of Virulent Pneumococcus Type I.

Monkey No.	Weight.	Apr. 29. Serum tests with Pn. III.		Apr. 30. Broth culture of Pn. III intra- trache- ally.	May 3. X-ray.	Result.	Autopsy.	Autopsy cultures	
		Agglu- tina.	Protec- tion.					Lung.	Heart's blood.
7	gm. 1,430	0	0	cc. 0.1	Negative.	Temporary febrile re- action; no pneumo- nia.			
82	732			0.1	Shadow, R. U.	Clinical pneumo- nia. Cri- sis on 7th day. Killed.	Interstitial pneumo- nia, R. U	Sterile.	Sterile.

Experiment 7.—Apr. 30, 1919. About 4 weeks after the test against *Pneumococcus* Type II, Monkey 7, vaccinated with living culture of *Pneumococcus* Type I, and Monkey 82, control, were injected intratracheally with 0.1 cc. of broth culture of a virulent *Pneumococcus* Type III. The results are shown in Table VI and Text-fig. 7.

The vaccinated monkey reacted to the injection of *Pneumococcus* Type III with a sharp but temporary rise of temperature, and a slight increase in leucocytes. The blood culture remained sterile. The monkey appeared sick during the afternoon of the day on which it was injected, but the next morning it was lively and well and remained so. The x-ray was negative, and if any pneumonia developed it must



TEXT-FIG. 7, *a* and *b*. Partial cross-immunity against *Pneumococcus* Type III following vaccination with living virulent *Pneumococcus* Type I. (*a*) Monkey 7; received 0.001 cc. of living culture of *Pneumococcus* Type I subcutaneously. (*b*) Monkey 82; control.

have been an exceedingly small patch. On the other hand, the control monkey developed a definite but rather mild attack of Type III pneumonia, with moderate leucocytosis and positive blood culture. The control recovered by crisis on the 7th day and autopsy showed a resolving interstitial pneumonia.

Vaccination with a living virulent *Pneumococcus* Type I apparently gave Monkey 7 a certain amount of cross-immunity against *Pneumococcus* Type III as well as against Type II. As with *Pneumococcus* Type II, the monkey possessed no demonstrable protective bodies in the blood against *Pneumococcus* Type III.

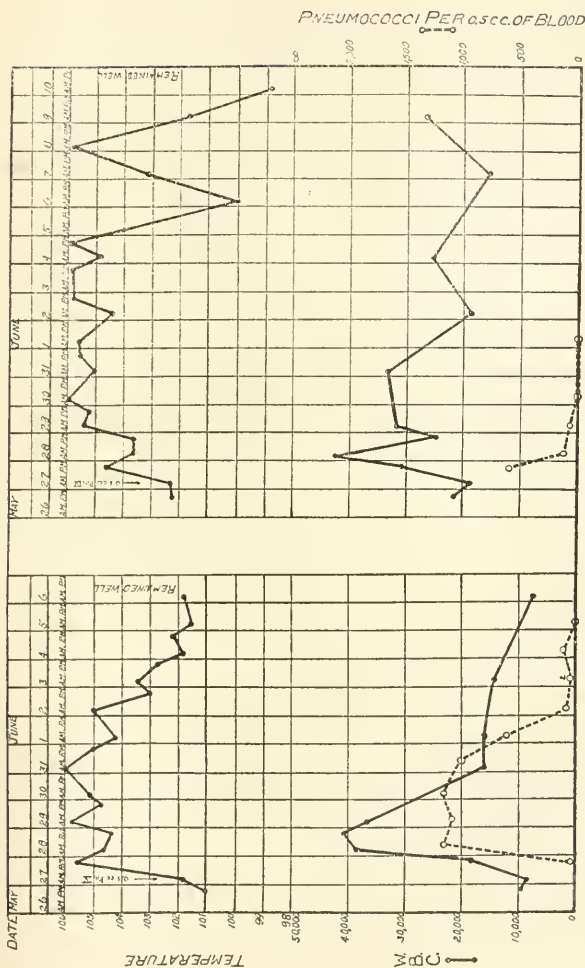
Test for Cross-Immunity against Pneumococcus Type IV Following Vaccination with Living Virulent Pneumococcus Type I.—One of the

TABLE VII.

Experimental Pneumococcus Type IV Pneumonia Following Vaccination with a Living Culture of Virulent Pneumococcus Type I.

Monkey No.	Weight.	May 27. Broth culture of Pn. IV intra-tracheally.	June 5. X-ray.	Result.	Autopsy.	Autopsy cultures.	
						Lung.	Heart's blood.
7	gm. 1,430	cc. 0.1	Negative.	Clinical pneumonia. Recovery by lysis on 8th day. Killed on 14th day.	Resolving lobar pneumonia, R. L., L. L.	No growth.	No growth.
111	2,219	0.1	Shadow, R. L.	Clinical pneumonia. Recovery on 14th day.			

vaccinated monkeys (No. 7) had resisted *Pneumococcus* Type I, Type II, and probably Type III pneumonia. It remained, therefore, only to test it against *Pneumococcus* Type IV. In this test a strain of Type IV was employed that had been recently isolated from a case of spontaneous *Pneumococcus* Type IV pneumonia in one of the stock monkeys (No. 97).



EXT-FIG. 8, a and b. Pneumococcus Type IV pneumonia following vaccination with living virulent Pneumococcus Type I. (a) Monkey 7; received 0.001 cc. of living culture of Pneumococcus Type I subcutaneously. (b) Monkey 111; control.

Experiment 8.—May 27, 1919. 4 weeks after being tested for immunity against *Pneumococcus* Type III, Monkey 7, vaccinated, and Monkey 111, control, were injected intratracheally with 0.1 cc. of broth culture of *Pneumococcus* Type IV (Strain M 97). Table VII and Text-fig. 8 show the results of the experiment.

Both monkeys developed Type IV pneumonia with high temperature, leucocytosis, pneumococcus septicemia, and final recovery; but the vaccinated monkey was well on the 8th day, while the control did not recover until the 14th day.

Apparently, there was not enough cross-immunity to protect the vaccinated animal against pneumonia, though there was enough, as with Monkey 48 in Experiment 6, to moderate and shorten somewhat the course of the disease. Just why Monkey 7 resisted the three fixed types of pneumococcus and then became infected with a *Pneumococcus* Type IV it is hard to say. Nearly 3 months had elapsed from the day of vaccination to the day when the monkey was injected with *Pneumococcus* Type IV, and it may be that by that time the immunity curve was on the downward way. Moreover, the pneumococcus used had recently been isolated from another infected monkey, and was therefore adapted, in a sense, to the species. The fixed strains of pneumococcus used had never been passed through monkeys.

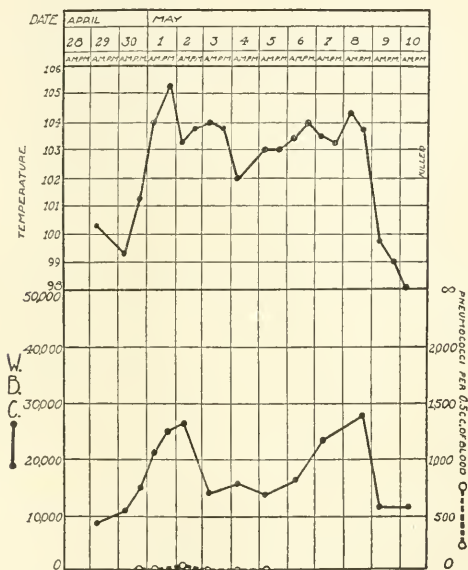
Spontaneous Pneumococcus Type IV Pneumonia Following Vaccination with a Living Virulent Pneumococcus Type I.—The following case corroborates the evidence obtained in the last experiment; namely, that there is not much cross-protection against *Pneumococcus* Type IV pneumonia in monkeys following vaccination with a living virulent culture of *Pneumococcus* Type I.

Experiment 9.—Monkey 47. *Macacus syrichtus*, male; weight 2,170 gm. Apr. 15, 1919, 10 a.m. Vaccinated with 0.001 cc. of 18 hour broth culture of *Pneumococcus* Type I (see Experiment 2). Apr. 30. Well and active. May 1. Looks sick; rapid labored respiration. May 2. Blood culture, *Pneumococcus* Type IV. May 3. Bronchial breathing in left axilla. May 4. Dyspnea persists; cough. May 5. Condition the same. May 9. Crisis and recovery. May 10. Killed.

Autopsy.—Resolving lobar pneumonia, left middle and lower lobes and right middle lobe; acute fibrinous pleuritis, left.

Text-fig. 9 shows the temperature, leucocyte, and blood culture curves of this case of spontaneous pneumonia. The disease ran a typical course, very similar to that seen in the experimental pneumo-

nia of monkeys. The blood, however, remained practically sterile (only one positive blood culture), and this fact suggests that here as in Monkey 7, *Pneumococcus* Type I vaccination afforded enough cross-immunity against *Pneumococcus* Type IV to moderate the severity of the pneumonia caused by the latter organism.

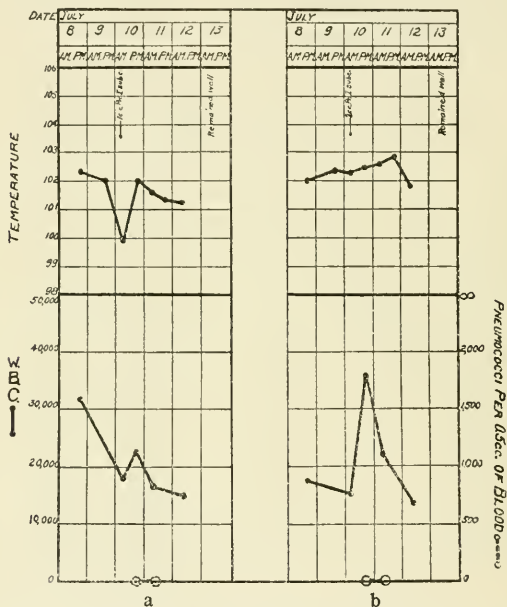


TEXT-FIG. 9. Monkey 47. Spontaneous *Pneumococcus* Type IV pneumonia in a monkey vaccinated with living virulent *Pneumococcus* Type I. Received 0.001 cc. of living culture of virulent *Pneumococcus* Type I subcutaneously.

*Vaccination with a Living Avirulent Culture of *Pneumococcus*.*

Up to this point all the experiments reported have shown the results obtained by vaccination with a living virulent pneumococcus. The remainder of the vaccination experiments were carried out with

a living avirulent pneumococcus, in order to determine how important a factor virulence was, on the one hand, and, on the other, how much depended upon the use of living or killed cultures.



TEXT-FIG. 10, *a* and *b*. Reactions following the subcutaneous inoculation of living avirulent *Pneumococcus* Type I. (*a*) Monkey 117; received 1 cc. of living culture subcutaneously. (*b*) Monkey 118; received 2 cc. of living culture subcutaneously.

The culture used for vaccination in the following experiments was an old stock *Pneumococcus* Type I which was avirulent for mice in doses of 1 cc. of broth culture. This strain was used for the preparation of the vaccine in the study of vaccination with killed cultures.⁸

⁸ Blake, F. G., and Cecil, R. L., *J. Exp. Med.*, 1920, xxxi, 499.

It was assumed that in view of the lack of virulence a much larger dose of vaccine would be necessary in order to obtain results; consequently, a dose of 1 to 2 cc. of broth culture was employed. The injections were given subcutaneously in the abdominal wall as in the previous experiments.

Experiment 10.—July 10, 1919. Two *Macacus syrichtus* monkeys (Nos. 117 and 118) were injected subcutaneously with an 18 hour broth culture of living avirulent *Pneumococcus* Type I. Monkey 117 received 1 cc. of culture (about 300 million pneumococci). Monkey 118 received 2 cc. (about 600 million pneumococci). The reactions are shown in Text-fig. 10.

Monkey 117 developed a small area of induration at the site of inoculation but had no constitutional reaction whatever. Monkey 118 also showed a slight local reaction, and in addition a moderate rise in leucocytes. The blood remained sterile in both monkeys.

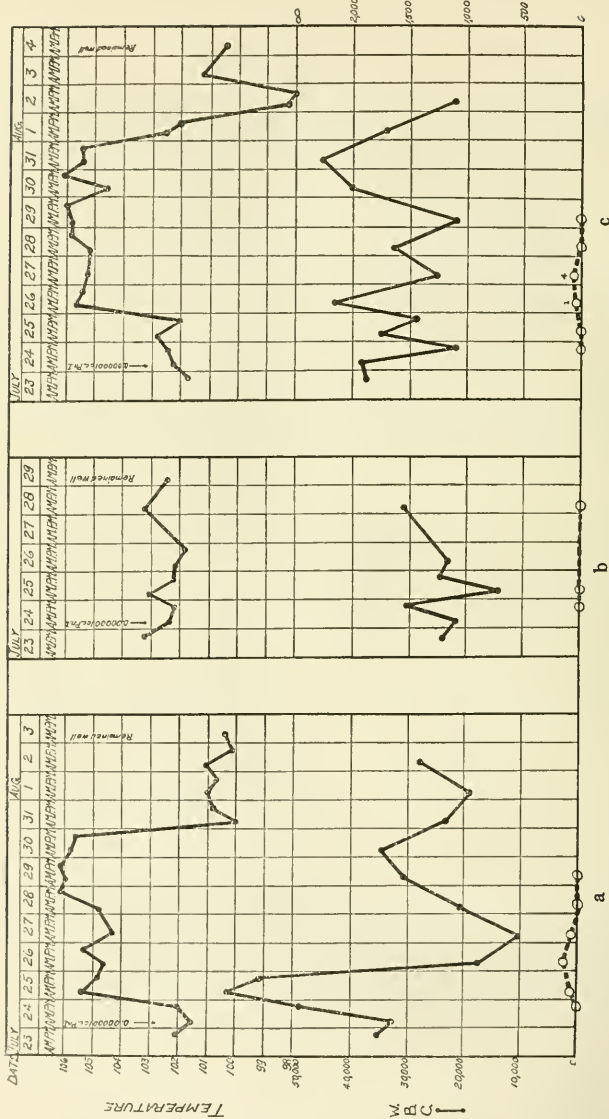
TABLE VIII.

Active Immunity Following Subcutaneous Injection of a Living Culture of Avirulent Pneumococcus Type I.

Monkey No.	Weight.	July 10. Broth culture of Pn. I subcutaneously.	Reaction.	July 24. Serum tests with Pn. I.		July 24. Broth culture of Pn. I intratracheally.	Result.
				Agglutins.	Protection.		
	gm.	cc.				cc.	
117	2,565	1	Slight; local.	0	0	0.000001	Clinical pneumonia. Recovery by crisis on 8th day.
118	2,915	2	Slight; local.	0	0	0.000001	Remained well.
127 (control).	2,700	0				0.000001	Clinical pneumonia. Recovery by crisis on 9th day.

This experiment indicated that living avirulent pneumococci could be administered subcutaneously in fairly large doses to monkeys without producing any serious local or general reaction.

The following experiment was carried out for the purpose of testing the amount of active immunity produced by vaccination with living avirulent pneumococci.



TEXT-FIG. 11. *a, b, and c.* Active immunity (Monkey 118) against *Pneumococcus* Type I following vaccination with 2 cc. of living avirulent *Pneumococcus* Type I; 1 cc. of the same culture failed to protect Monkey 117. *(a)* Monkey 117; received 1 cc. of living avirulent culture subcutaneously. *(b)* Monkey 118; received 2 cc. of living avirulent culture subcutaneously. *(c)* Monkey 127; control.

Experiment 11.—July 24, 1919. Monkeys 117 and 118, both vaccinated on July 10 with living avirulent pneumococci, and Monkey 127, control, were injected intratracheally with 0.000001 cc. of broth culture of *Pneumococcus* Type I (Table VIII). Monkey 117, which received the small dose (1 cc.) of living vaccine, developed a mild pneumonia and recovered by crisis on the 8th day of the disease. Monkey 118, which received the large dose (2 cc.) of living vaccine, remained perfectly well. The control (Monkey 127) developed pneumonia and ran a typical course with crisis on the 9th day.

Text-fig. 11 shows the temperature, leucocyte, and blood culture curves. Monkey 118 presented no reaction of any kind. Monkey 117 ran a typical lobar pneumonia, with positive blood culture, high leucocytosis, and crisis on the 8th day. Monkey 127 also ran a typical course with crisis on the 9th day.

Living avirulent pneumococci, when injected subcutaneously, appear to excite an immunity equal in degree to that produced by living virulent pneumococci, if a large enough dose is administered. In this experiment 1 cc. of broth culture of living avirulent pneumococci did not confer a sufficiently high degree of immunity to protect the monkey from pneumonia. When 2 cc., however, of the same culture were administered to another monkey, the protection was satisfactory. In other words, the dose is a factor to be considered in vaccinating with living avirulent pneumococci.

DISCUSSION.

The inferences to be drawn from the experiments reported in this study are plain. A high degree of immunity against pneumococcus pneumonia can be induced by the subcutaneous injection of living virulent pneumococci, but the method is too dangerous for any sort of practical application. Vaccination with attenuated living pneumococci could probably be practised with impunity, but the problem of transporting and keeping alive large quantities of pneumococci in the field would be difficult to solve. The fact that a higher degree of immunity is produced by living pneumococci than by dead pneumococci is not surprising in the light of previous observations on vaccination with other bacteria. The degree of cross-immunity, however, which sometimes followed vaccination with living *Pneumococcus* Type I was surprising and confirms the fact already established that the various types of pneumococci are closely related biologically.

This study had suggested a number of theoretical problems. In the first place, why do living pneumococci confer a more efficient immunity than dead pneumococci? Virulence appears to play some part, but even with avirulent strains good protection can be secured with sufficiently large doses. These animals vaccinated with living cultures are, of course, in a sense infected, and we are accustomed to think of infection as bestowing a more efficacious immunity than mere vaccination with killed cultures.

The question also arises as to the significance of agglutinins and the so called protective bodies in an animal's blood. It has been shown in the experiments reported that the serum of a monkey may be entirely free from these substances, and yet the animal may possess a high grade of immunity against pneumonia. On the other hand, it was pointed out in the study of pneumococcus saline vaccines¹ that the serum of a vaccinated monkey might protect mice against 100 or even 1,000 minimal lethal doses of pneumococci, and still that monkey be susceptible to experimental pneumococcus pneumonia. These facts complicate the whole question of resistance to pneumococcus infection and revive the old problem of humoral *versus* cellular immunity.

These studies also emphasize the fact that "immunity" is a relative term and under any circumstances dependent on a number of factors. Every immunologist is familiar with the great differences which animals manifest in their capacity to produce antibodies. Even with the most ideal methods of vaccination there will always be certain animals that respond poorly to vaccine, and these animals will not possess the same amount of immunity that others will have.

Furthermore, the degree of immunity against a certain microorganism depends in large measure on the virulence of the strain used in testing it. For example, in the experiments on cross-immunity, Monkey 7 resisted infection with *Pneumococcus* Type III which was not very virulent for monkeys, but could not resist *Pneumococcus* Type IV which, in this particular instance, happened to be virulent for monkeys. In view of this fact the question arises whether in the experiments with *Pneumococcus* Type I lipovaccine and saline vaccine immunity would not have been demonstrated if a less virulent strain of *Pneumococcus* Type I had been used for testing the efficacy of the vaccine.

All that has been said regarding virulence applies with equal force to the size of the infecting dose. Even in well immunized monkeys experimental pneumonia could probably have been induced if a sufficiently large dose of culture had been injected into the trachea. This would have been a most artificial procedure, but the argument holds in spite of that fact. The animal possesses a definite sum of resistance, and when that sum is spent it becomes at once susceptible to infection.

CONCLUSIONS.

1. The subcutaneous injection of small doses of living virulent *Pneumococcus* Type I stimulates in monkeys a degree of active immunity sufficient to protect them against experimental pneumococcus pneumonia of homologous type.

2. The subcutaneous injection of living avirulent *Pneumococcus* Type I, if administered in a sufficiently large dose, likewise renders the monkey immune to a subsequent pneumonia of homologous type.

3. Vaccination of monkeys with small doses of living virulent pneumococci may or may not be followed by a severe constitutional reaction, depending on the natural resistance of the individual. The severe reactions are caused by the development of a pneumococcus septicemia, which is either temporary, or leads to a fatal termination. The mild reactions are not accompanied by septicemia, and there are no symptoms other than a slight elevation of temperature and moderate leucocytosis. Vaccination with living avirulent pneumococci does not induce severe reactions and is not accompanied by pneumococcus septicemia.

4. Active immunity against pneumococcus pneumonia, produced by vaccination with living pneumococci, appears to be largely independent of the presence or absence of agglutinins and protective bodies in the serum of the monkey.

5. Vaccination with living cultures of *Pneumococcus* Type I confers against other types of pneumococci a certain amount of cross-immunity which, however, varies considerably with the individual monkey.

6. Immunity against pneumococcus, like other forms of immunity, is a relative term, and depends upon the capacity of the individual for antibody production, the virulence of the invading microorganism, and the size of the dose injected.

STUDIES ON EXPERIMENTAL PNEUMONIA.

VI. ACTIVE IMMUNITY FOLLOWING EXPERIMENTAL PNEUMOCOCCUS PNEUMONIA IN MONKEYS.

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The tendency of certain individuals to repeated attacks of pneumonia has at times given rise to doubt whether acquired immunity to pneumonia exists. Considerable evidence, however, can be brought forward to show that a rather high degree of immunity to the pneumococcus follows an attack of pneumonia. The crisis itself is a striking expression of immunity. Furthermore, Dochez¹ has shown that the serum of patients convalescing from pneumonia usually contains protective substances against the homologous type of pneumococcus, and Blake² has demonstrated precipitins in the serum of cases of pneumonia that terminate favorably. Aside from these clinical studies, however, accurate information on the subject of immunity following pneumonia is meager.

The experiments herewith reported were performed for the purpose of testing the degree of active immunity in monkeys subsequent to an attack of experimental pneumococcus pneumonia.

Usually the monkey has first been subjected to a pneumonia of one of the four pneumococcus types; and following this, the resistance to reinfection with a pneumococcus of the same type has been tested. Then, as in the study of vaccination with living pneumococci (Paper V), tests for cross-immunity against one or more of the other types of pneumococcus have been carried out. The same virulent pneumococci were used in these experiments as in the previous studies and the technique used for producing experimental pneumonia was identical with that already described. Philippine monkeys (*Macacus syrichtus*) were employed throughout.

¹ Dochez, A. R., *J. Exp. Med.*, 1912, xvi, 665.

² Blake, F. G., *Arch. Int. Med.*, 1918, xxi, 779.

*Active Immunity Following Experimental Pneumococcus Type I
Pneumonia.*

The first experiment was a test for active immunity following experimental *Pneumococcus* Type I pneumonia. It was shown in Paper I³ that experimental pneumonia in monkeys is usually fatal when a highly virulent pneumococcus is used for producing the disease. By using very minute infecting doses, however, a certain number of recoveries can be obtained. In a series of *Macacus* monkeys

TABLE I.
Experimental Lobar Pneumonia.

Monkey No.	Weight.	Apr. 29. Broth culture of Pn. I in- tratracheally.	May 15. X-ray.	Result.
	<i>gm.</i>	<i>cc.</i>		
75	4,200	0.0001	Shadow, R. L., L. L.*	Clinical pneumonia. Recovery by crisis on 12th day.
77	2,600	0.000001	Shadow, L. L.	Clinical pneumonia. Recovery by lysis on 11th day.

* R. L., R. M., R. U., etc., indicate lobes of the lung. The cardiac lobe is included as part of the right lower lobe.

inoculated intratracheally with a virulent *Pneumococcus* Type I, two recovered. The following experiment gives the record of these two monkeys.

Experiment 1.—Apr. 29, 1919. Monkey 75 received 0.0001 cc. and Monkey 77, 0.000001 cc. of broth culture of *Pneumococcus* Type I intratracheally. Both monkeys promptly developed symptoms and signs of lobar pneumonia, with positive blood cultures and sharp leucocyte reactions (Table I, Text-fig. 1). Both monkeys made uneventful recoveries.

2 weeks after the recovery of Monkeys 75 and 77, their blood was tested for agglutinins and protective bodies. Monkey 77 exhibited no agglutinins, but did show some protective bodies; the mouse that received 0.0000001 cc. of culture lived 4 days, and the mouse receiving 0.000001 cc. lived 3 days.

³ Blake, F. G., and Cecil, R. L., *J. Exp. Med.*, 1920, xxxi, 403.

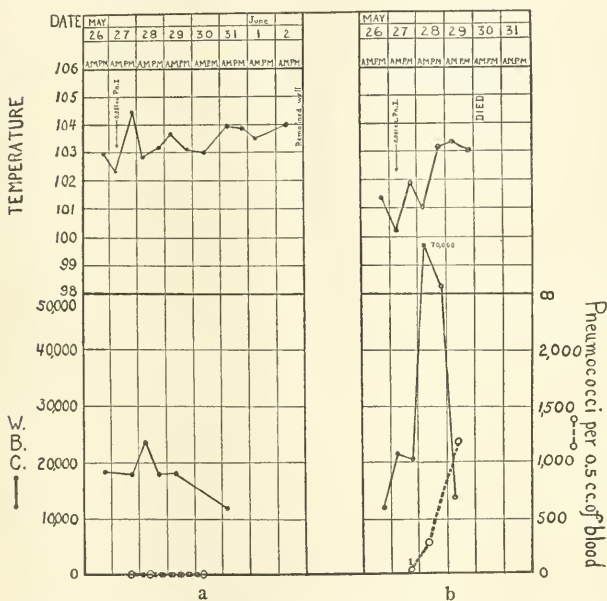
TABLE II.
Active Immunity Following Experimental Lobar Pneumonia.

Monkey No.	Weight. <i>gm.</i>	May 27. Serum tests with Pn. I.			May 27. Broth culture of Pn. I in- tratracheally.	Result.	Autopsy.	Autopsy cultures.	
		Agglu- tins.	Protection.	Control.				Lung.	Heart's Pericar- dium.
75	4,200	0	0		<i>cc.</i> 0.001	Remained well. No signs of pneu- monia.			
77	2,600	0	0.00001 cc. D.* 48 hrs. 0.000001 cc. D. 3 days. 0.0000001 cc. D. 4 days.	0.00001 cc. D. 48 hrs. 0.000001 cc. D. 48 hrs. 0.0000001 cc. D. 48 hrs.	0.000001	Remained well. No signs of pneu- monia.			
109 (con- trol).	4,585				0.001	Clinical pneumonia. D. 3rd day.	Lobar pneumonia; stage of engorge- ment, R. U., L. U., R. M., L. M.	Pn. I	Pn. I
110 (con- trol).	2,710				0.000001	Clinical pneumonia. D. 7th day.	Lobar pneumonia; mixed red and gray stages, entire right lung; acute peri- carditis.	" I	" I Pn. I

* D. indicates died; S., survived.

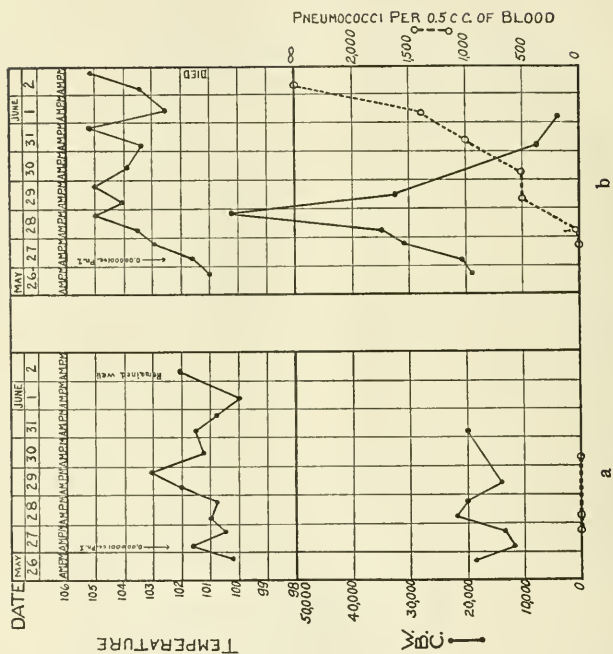
The next step was to test these two monkeys for active immunity against *Pneumococcus* Type I.

Experiment 2.—May 27, 1919. Nearly 3 weeks after their recovery from pneumonia, Monkeys 75 and 77, with two *Macacus* controls were injected intra-tracheally with a broth culture of virulent *Pneumococcus* Type I (the same



TEXT-FIG. 2, *a* and *b*. Active immunity against *Pneumococcus* Type I following *Pneumococcus* Type I pneumonia. (*a*) Monkey 75; immunity following pneumonia. (*b*) Monkey 109; control.

strain with which they had been previously infected). Monkey 75 and its control, Monkey 109, each received 0.001 cc. of culture; Monkey 77 and its control, Monkey 110, each received 0.000001 cc. Table II and Text-figs. 2 and 3 show the results obtained.



TEXT-FIG. 3, a and b. Active immunity against Pneumococcus Type I following Pneumococcus Type I pneumonia. (a) Monkey 77; immunity following pneumonia. (b) Monkey 110; control.

In Table II it will be seen that the two monkeys which had had a previous attack of *Pneumococcus* Type I pneumonia remained well, while the two controls developed *Pneumococcus* Type I pneumonia. Monkey 75 (Text-fig. 2) showed a very slight temporary rise in temperature and leucocytes, but was lively and well and displayed no symptoms whatever of pneumonia. The rather high average temperature was attributed at the time to the hot weather, but subsequently autopsy showed a chronic pulmonary tuberculosis. Monkey 109, the control to Monkey 75, was overwhelmed by the large dose (0.001 cc.) of culture injected and died on the 3rd day of the illness with lobar pneumonia and *Pneumococcus* Type I septicemia.

Monkey 77 (Text-fig. 3) also remained well and showed very little temperature or leucocyte reaction. The control, Monkey 110, developed lobar pneumonia and died on the 7th day of the disease with a heavy *Pneumococcus* Type I septicemia.

These experiments corroborate the evidence already at hand in favor of a high degree of active immunity following pneumococcus pneumonia. It will be noted that this immunity existed in Monkey 75 without any evidence of agglutinins or protective substances in the blood. The amount of protective substance in Monkey 77 was almost negligible, yet it also was able to resist reinfection.

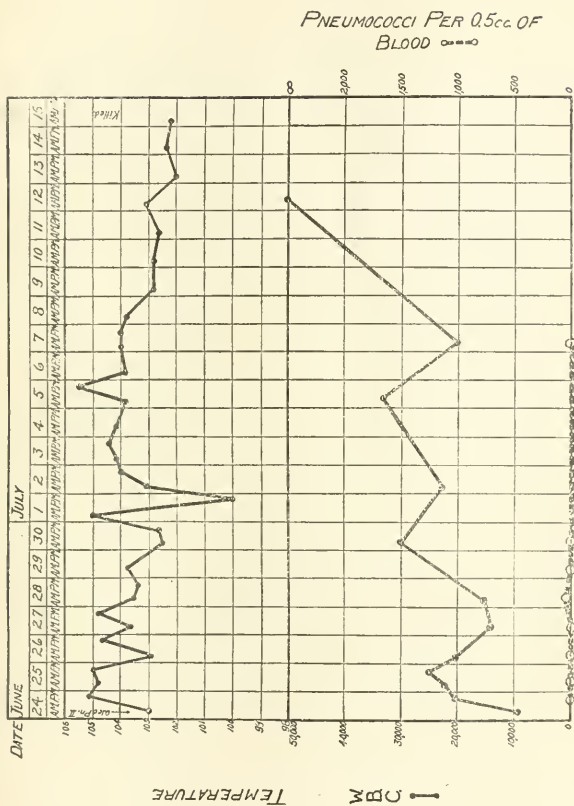
Tests for Cross-Immunity Following Pneumococcus Type I Pneumonia.

In the preceding paper it has been shown that vaccination with a living culture of *Pneumococcus* Type I conferred a high degree of immunity against pneumonia of the homologous type; and furthermore, that by such vaccination a certain but variable amount of cross-immunity was also established against the other types of pneumococcus pneumonia. It was therefore desirable to determine whether a similar cross-immunity could be demonstrated in monkeys that had survived an attack of *Pneumococcus* Type I pneumonia.

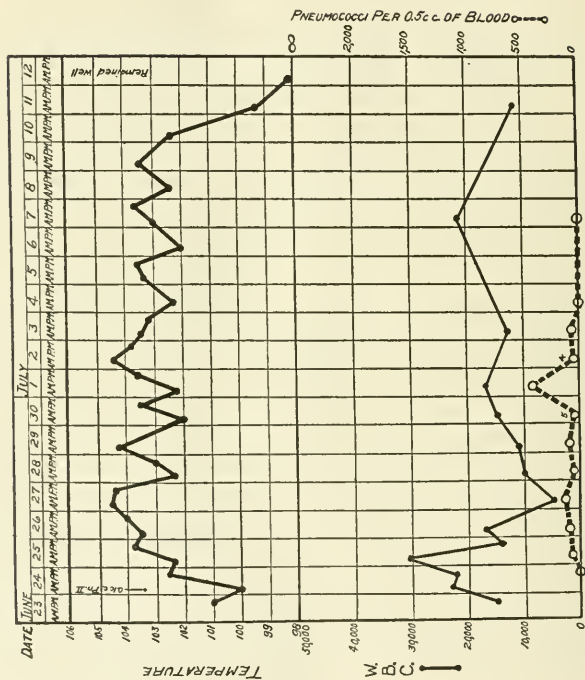
Test for Cross-Immunity against Pneumococcus Type II Following Pneumococcus Type I Pneumonia.—In the following experiment one of the monkeys (No. 75) that resisted reinfection with *Pneumococcus* Type I was tested for cross-immunity against *Pneumococcus* Type II. The same virulent strain of *Pneumococcus* Type II was

TABLE III.
Pneumococcus Type II Pneumonia Following Pneumococcus Type I Pneumonia.

Monkey No.	Weight.	Apr. 29-May 10.	May 27.	June 23. Serum tests with Pn. II.		June 24. Broth culture of Pn. II intra-tracheally.	Result.	Autopsy.	Autopsy cultures.	
				Agglutins.	Protection.				Lung.	Heart's blood.
75	3,862 gm.	Pn. I pneumonia.	Resisted reinfection with Pn. I.	0	0.000001 cc. D. 60 hrs. 0.00001 cc. S. 0.0001 cc. D. 18 hrs. 0.001 cc. D. 18 hrs.	0.1 cc. D. 48 hrs. 0.00001 cc. D. 20 hrs. 0.0001 cc. D. 18 hrs.	Clinical pneumonia. Crisis on 8th day. Continuous temporary temperature. Killed on 22nd day.	Chronic pulmonary tuberculosis; general military tuberculosis.	No pneumococci.	No pneumococci.
91 (control).	4,002					0.1	Clinical pneumonia. Recovery on 18th day.			



TEXT-FIG. 4. Monkey 75. Pneumococcus Type II pneumonia subsequent to Pneumococcus Type I pneumonia. Pneumococcus Type I pneumonia from Apr. 29 to May 10.



TEXT-FIG. 5. Monkey 91; control for Monkey 75. Experimental Pneumococcus Type II pneumonia.

used in this experiment that had been employed in the vaccination experiments.

Experiment 3.—June 24, 1919. 6 weeks after the attack of *Pneumococcus* Type I pneumonia, Monkey 75 received 0.1 cc. of broth culture of *Pneumococcus* Type II intratracheally. Monkey 91 served as control, and received the same dose of culture. The results are shown in Table III and Text-figs. 4 and 5.

Both monkeys developed pneumonia and ran rather unusually long courses with final recovery. Monkey 75, however, continued to run a slight temperature and was killed July 15, the 22nd day after inoculation. Autopsy showed a chronic pulmonary tuberculosis, with early miliary tuberculosis of the various organs. Monkey 91, the control, had had an attack of spontaneous *Pneumococcus* Type IV pneumonia several months before the experiment, so was not an ideal control. It was the only one available, however, at this time, and had to be used. Ordinarily, an infecting dose as large as the one used in this experiment (0.1 cc.) would have been sufficient to kill a control.

The experiment shows that an attack of *Pneumococcus* Type I pneumonia did not afford enough cross-immunity to protect Monkey 75 from infection with *Pneumococcus* Type II. How much of a factor the tuberculosis was in this animal and to what extent it affected the immunity it is impossible to say. The tuberculous process was probably active at the time the immunity against *Pneumococcus* Type I was tested, for the animal was then running an abnormally high temperature; yet the presence of tuberculosis apparently did not lessen the degree of resistance to *Pneumococcus* Type I.

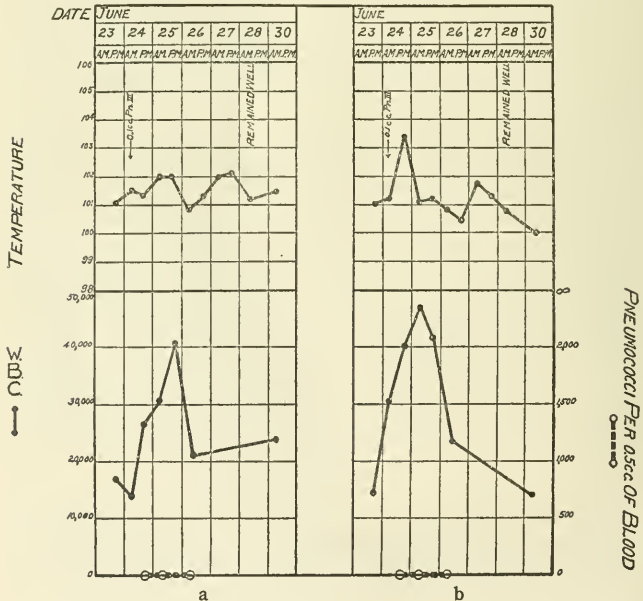
Tests for Cross-Immunity against Pneumococcus Type III Following Pneumococcus Type I Pneumonia.—The next step in this series of experiments was to test a monkey that had recovered from *Pneumococcus* Type I pneumonia for cross-immunity against *Pneumococcus* Type III. Monkey 77 was selected for this test.

Experiment 4.—June 24, 1919. 6 weeks after recovery from *Pneumococcus* Type I pneumonia, Monkey 77 received 0.1 cc. of broth culture of virulent *Pneumococcus* Type III intratracheally. The control, Monkey 115, received the same dose at the same time. Table IV and Text-fig. 6 show the results obtained.

TABLE IV.

Inoculation with Pneumococcus Type III Following Pneumococcus Type I Pneumonia.

Monkey No.	Weight.	Apr. 29-May 9.	May 27. Broth culture of Pn. I intratracheally.	June 23. Serum tests with Pn. III.		June 24. Broth culture of Pn. III intratracheally.	Result.
				Agglutinations.	Protection.		
77	gm. 2,680	Experimental Pn. I pneumonia.	Resisted reinfection with Pn. I.	0	0	cc. 0.1	No symptoms of pneumonia.
115 (control).	3,015					0.1	Temporary febrile reaction with accelerated respiration and leucocytosis.



TEXT-FIG. 6, *a* and *b*. Inoculation with *Pneumococcus* Type III following *Pneumococcus* Type I pneumonia. (*a*) Monkey 77; *Pneumococcus* Type I pneumonia from Apr. 29 to May 9. (*b*) Monkey 115; control.

Monkey 77 remained well and had no symptoms whatever except a moderate rise in leucocytes. The temperature of the control monkey rose to 103.4° F. and there was a sharp leucocyte reaction, but the blood culture remained sterile and the monkey did not appear very sick. Evidently the control suffered nothing more than a bronchitis or a very small patch of consolidation.

The *Pneumococcus* Type III strain used in this experiment at no time proved highly virulent for monkeys, though 0.000001 cc. of a broth culture would invariably kill a mouse. It seemed advisable to repeat this test on another monkey using a larger infecting dose of *Pneumococcus* Type III. Accordingly, another monkey (No. 96) that had recovered from *Pneumococcus* Type I pneumonia was tested against *Pneumococcus* Type III. Monkey 96 had *Pneumococcus* Type I pneumonia from May 13 to 20.⁴

Experiment 5.—July 1, 1919. Monkey 96, about 6 weeks after the attack of *Pneumococcus* Type I pneumonia, received 1 cc. of broth culture of *Pneumococcus* Type III intratracheally (Table V, Text-fig. 7). The control, Monkey 107, likewise received 1 cc. of the same culture intratracheally.

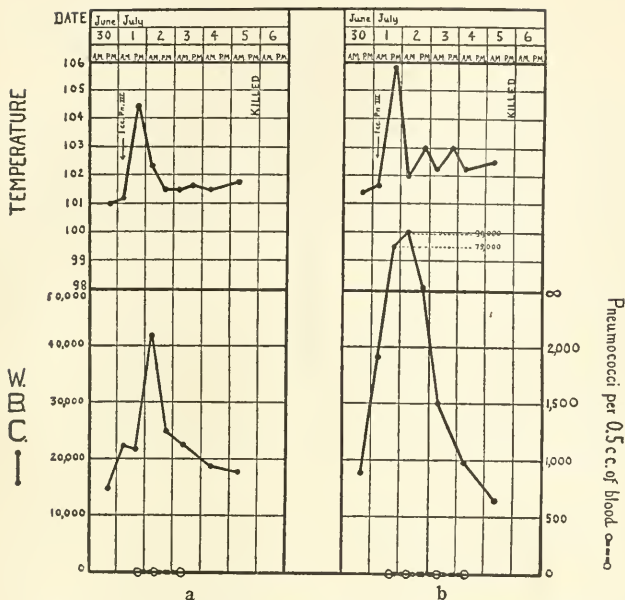
By referring to Table V it will be seen that both monkeys developed a mild interstitial pneumonia, the symptoms of which persisted only 48 hours. Neither monkey presented a positive blood culture at any time. The temperature and leucocyte curves for the two monkeys were strikingly alike.

It cannot be maintained that the vaccinated monkey in this experiment showed any definite cross-immunity against *Pneumococcus* Type III. The disease ran a mild course in both animals for the probable reason that this particular strain of *Pneumococcus* Type III though virulent for mice possessed only slight virulence for monkeys.

⁴ Blake, F. G., and Cecil, R. L., *J. Exp. Med.*, 1920, xxxi, 530, Experiment 3.

TABLE V.
Pneumococcus Type III Pneumonia Following Pneumococcus Type I Pneumonia.

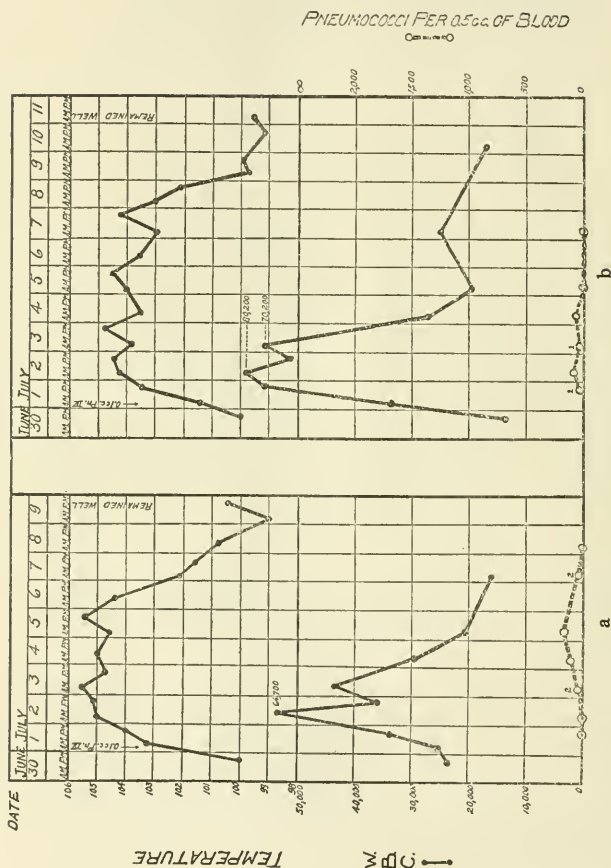
Monkey No.	Weight. <i>gm.</i>	May 13. Broth culture of Pn. I in- tratracheally. <i>cc.</i>	Result.	July 1. Broth culture of Pn. III in- tratracheally. <i>cc.</i>	Result.	Autopsy.	Autopsy cultures.	
							Lung.	Heart's blood.
96	2,655	0.000001	Clinical pneu- monia. Crisis on 8th day.	1	Very mild pneu- monia. Killed on 5th day.	Interstitial pneu- monia, R. U., L. U.	No growth.	No growth.
107 (control).	2,334			1	Very mild pneu- monia. Killed on 5th day.	Interstitial pneu- monia, R. L.	" "	" "



TEXT-FIG. 7, *a* and *b*. Pneumococcus Type III pneumonia subsequent to Pneumococcus Type I pneumonia. (*a*) Monkey 96; Pneumococcus Type I pneumonia from May 13 to 20. (*b*) Monkey 107; control.

Tests for Cross-Immunity against Pneumococcus Type IV Following Pneumococcus Type I Pneumonia.—It has been shown in the previous paper that vaccination with a living Pneumococcus Type I gave no appreciable cross-protection against Pneumococcus Type IV pneumonia. In the present study a somewhat similar test was made for cross-immunity against Pneumococcus Type IV following Pneumococcus Type I pneumonia.

Experiment 6.—July 1, 1919. Monkey 77, 7 weeks after the attack of Pneumococcus Type I pneumonia, and the control, Monkey 115, each received 0.1



TEXT-FIG. 8, *a* and *b*, Pneumococcus Type IV pneumonia subsequent to Pneumococcus Type I pneumonia. (*a*) Monkey 77; Pneumococcus Type I pneumonia from Apr. 29 to May 9. (*b*) Monkey 115; control.

cc. of *Pneumococcus* Type IV intratracheally (Table VI). This was a virulent pneumococcus isolated from a case of spontaneous lobar pneumonia (Monkey 97) and killed a mouse in doses of 0.000001 cc. of broth culture.

Both monkeys proved susceptible and ran typical courses of lobar pneumonia (Text-fig. 8). Monkey 77 recovered by crisis on the 7th day, Monkey 115 on the 9th day. Both animals had very pronounced leucocyte reactions and positive blood cultures.

These two cases were about equally severe. As both recovered, it is impossible to say whether Monkey 77 had any cross-immunity or not, but the evidence is against such a hypothesis.

TABLE VI.

Inoculation with Pneumococcus Type IV of the Two Monkeys Tested in Experiment 4.

Monkey No.	July 1. Serum tests with Pn. IV (Strain M 97).		July 1. Broth culture of Pn. IV (Strain M 97), intratracheally.	Result.
	Agglu- tinins.	Protection.		
77	0	0	cc. 0.1	Clinical pneumonia. Recovery by crisis on 7th day.
115	0	0	0.1	Clinical pneumonia. Recovery by crisis on 9th day.

*Active Immunity Following an Attack of Pneumococcus Type IV
Pneumonia.*

The so called *Pneumococcus* Type IV is not a type in the true sense as it consists merely of a large number of pneumococcus strains which do not fall into the three fixed groups and which usually have no biological connection with one another. An animal immunized against one strain of *Pneumococcus* Type IV develops agglutinins and protective bodies for that particular strain, but as a general rule this serum fails to agglutinate or protect against other Type IV strains.

The question therefore naturally arose whether an attack of *Pneumococcus* Type IV pneumonia would confer immunity against a second attack by the same strain, and also whether an attack of

Pneumococcus Type IV pneumonia would confer any cross-immunity against other strains of Pneumococcus Type IV.

In order to answer these questions two monkeys that had survived attacks of Pneumococcus Type IV pneumonia were inoculated a second time with Pneumococcus Type IV as shown in the following experiment.

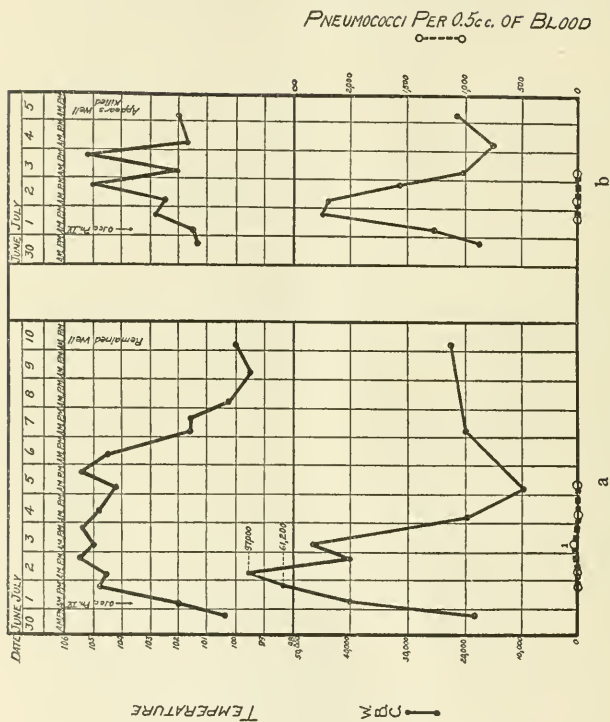
Experiment 7.—July 1, 1919. A large *Macacus*, Monkey 19, 3 weeks after an attack of spontaneous Pneumococcus Type IV pneumonia, was injected intratracheally with 0.1 cc. of broth culture of Pneumococcus Type IV, Strain M 97. At the same time Monkey 111, 3 weeks subsequent to an attack of experimental Pneumococcus Type IV pneumonia (Strain M 97) was also injected intratracheally with 0.1 cc. of broth culture of Pneumococcus Type IV, Strain M 97. Table VII and Text-fig. 9 show the results of this experiment.

Both monkeys developed Pneumococcus Type IV pneumonia and both recovered. Monkey 19 ran a typical mild course with only one positive blood culture. Monkey 111, inoculated a second time with the identical strain injected 1 month previously, developed an abortive pneumonia and was sick only 2 or 3 days. The blood culture in this animal was never positive.

An unexpected sequel to this experiment presented itself when a biological study was made of the various Pneumococcus Type IV strains that had been isolated during the epidemic of spontaneous pneumonia in the stock monkeys. It was found that the strain originally isolated from Monkey 19 during the attack of spontaneous pneumonia and Strain M 97 were agglutinated by the same Pneumococcus Type IV serum; in other words they belonged to the same biological group. The experiment had been planned to test the amount of immunity against Pneumococcus Type IV following an attack of Pneumococcus Type IV pneumonia. Monkey 19 was to be tested for cross-immunity against a supposedly heterologous strain, Monkey 111 for active immunity against the homologous strain. But since the pneumococcus isolated from Monkey 19 during its original attack of spontaneous pneumonia and Strain M 97 belong to the same subgroup of Pneumococcus Type IV, it is apparent that in both monkeys we were testing for active immunity against the homologous strain of Pneumococcus Type IV. Strangely enough both monkeys failed to show sufficient immunity to protect them against reinfection with

TABLE VII.
Reinfection with Pneumococcus Type IV Following an Attack of Pneumococcus Type IV Pneumonia.

Monkey No.	Weight.	Lobar pneumonia Pn. IV.	July 1: Serum tests with Pn. IV (Strain M 97).		July 1: Broth culture of Pn. IV (Strain M 97) intratracheally.	Result.	Autopsy.	Autopsy cultures.	
			Agglutinins.	Protections.				Lung.	Heart's blood.
19	4,000 gm.	June 1-8. Spontaneous (Strain M 19).	0	0	cc. 0.1	Clinical pneumonia. Recovery by crisis on 7th day.			
111	1,670	May 27-June 9. Experimental (Strain M 97).	0	0	0.1	Temporary febrile reaction and leucocytosis. Killed on 5th day.	Old unresolved pneumonia, R. U. Patch of fresh infection (engorgement), R. L.	R. L., Pn. IV.	No growth.



TEXT-FIG. 9, *a* and *b*. Pneumococcus Type IV pneumonia subsequent to Pneumococcus Type IV pneumonia. (*a*) Monkey 19; spontaneous Pneumococcus Type IV pneumonia from June 1 to 8. (*b*) Monkey 111; experimental Pneumococcus Type IV pneumonia (Strain M 97) from May 27 to June 9.

the same strain of *Pneumococcus* Type IV which had excited in them an attack of pneumonia a month previously. But although these monkeys were unable to resist reinfection with *Pneumococcus* Type IV, it is clear that one of them, Monkey 111, had a considerable amount of active immunity—enough to reduce the second attack to a very mild, abortive pneumonia. Furthermore, it is worthy of note that the autopsy on this monkey showed that it had never recovered completely from the first attack. An old unresolved pneumonia was found in the right upper lobe, while the fresh infection was limited to the right lower lobe.

Test for Cross-Immunity against Pneumococcus Type I Following Pneumococcus Type IV Pneumonia.—The last experiment in this series was undertaken to determine whether an attack of *Pneumococcus* Type IV pneumonia would confer any cross-immunity against *Pneumococcus* Type I pneumonia.

Experiment 8.—Monkey 115. *Macacus syrichtus*, female; weight, 3,015 gm. July 1, 1919. Intratracheal injection of 0.1 cc. of 18 hour broth culture of *Pneumococcus* Type IV, Strain M 97. July 2. Symptoms of pneumonia. Blood culture positive, *Pneumococcus* Type IV. July 9. Crisis and recovery. July 26. Intratracheal injection of 0.000001 cc. of 18 hour broth culture of *Pneumococcus* Type I. July 27. Symptoms of pneumonia. July 29. Blood culture positive (one colony of *Pneumococcus* Type I in 0.5 cc. of blood). Aug. 2. Crisis and recovery. The results are shown in Text-fig. 10.

This experiment is in line with the other experiments on cross-immunity. The monkey was not sufficiently immune to resist infection with *Pneumococcus* Type I, but, in view of the mild course and the practically sterile blood culture, it would appear that enough cross-immunity was present to modify favorably the course of the disease. Certainly, if an attack of *Pneumococcus* Type IV pneumonia does not stimulate enough protection to prevent a second infection by the same strain, it could hardly be expected to elaborate an adequate cross-immunity against a highly virulent *Pneumococcus* Type I.

monkeys. In both the Type II and Type III experiments, however, rather large infecting doses were employed, 0.1 cc. of *Pneumococcus* Type II and 0.1 to 1 cc. of *Pneumococcus* Type III. As in vaccination with living cultures, a small amount of cross-immunity against the other fixed types of pneumococcus is probably always present for a certain length of time following experimental *Pneumococcus* Type I pneumonia. As in vaccination with living cultures, however, the degree of cross-immunity depends to some extent on individual variation. The virulence and dose of the invading microorganism are, of course, other factors of great importance. An attack of *Pneumococcus* Type I pneumonia does not appear to induce any higher degree of cross-immunity than vaccination with a living *Pneumococcus* Type I affords.

It is impossible to say why *Pneumococcus* Type IV pneumonia fails to protect the monkey against a second infection by the homologous strain when *Pneumococcus* Type I confers excellent immunity against reinfection. The highly parasitic character of *Pneumococcus* Type I may account for this difference. At any rate, these observations offer little hope for a satisfactory prophylactic vaccine against *Pneumococcus* Type IV pneumonia.

From the information obtained in this study it would appear possible that individuals who suffer from repeated attacks of pneumonia are being reinfected from time to time with *Pneumococcus* Type IV. Further evidence in favor of such a theory is afforded by the fact that in cases of this kind the disease usually runs a mild course.

Attention is again invited to the inconsistency between tests for protective bodies against the pneumococcus in the serum of monkeys and tests for active immunity against pneumonia in these animals. In this respect the results are in harmony with those reported in Papers IV⁵ and V where striking disagreements were sometimes noted in the two methods for testing immunity. In the present study, for example, Monkey 75 showed no agglutinins or protective bodies against *Pneumococcus* Type I in the blood subsequent to the attack of *Pneumococcus* Type I pneumonia; yet when reinjected with *Pneumococcus* Type I intratracheally, this animal possessed a sufficient degree of active immunity to resist reinfection.

⁵ Blake, F. G., and Cecil, R. L., *J. Exp. Med.*, 1920, xxxi, 519.

CONCLUSIONS.

1. Experimental Pneumococcus Type I pneumonia in monkeys confers on them an immunity which protects them against subsequent infection with the homologous type of pneumococcus. The duration of this immunity has not been determined.

2. A certain amount of cross-immunity against the other fixed types of pneumococcus pneumonia may or may not be present following experimental Pneumococcus Type I pneumonia. The degree of cross-immunity is difficult to measure and probably varies widely with the individual monkey.

3. Experimental Pneumococcus Type IV pneumonia in monkeys confers slight if any protection against subsequent infection with the same, or with an homologous strain of Pneumococcus Type IV.

4. There is no evidence in monkeys of cross-immunity against Pneumococcus Type IV pneumonia following Pneumococcus Type I pneumonia; and conversely, Pneumococcus Type IV pneumonia confers no cross-immunity against Pneumococcus Type I pneumonia.

EXPERIMENTAL SYPHILIS IN THE RABBIT.

II. PRIMARY INFECTION IN THE SCROTUM.

PART 1. REACTION TO INFECTION.

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PLATES 72 TO 82.

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Primary skin lesions in the rabbit were first reported in 1908. Hoffmann, Löhe, and Mulzer (1), and Ossola (2) reported instances of scrotal infection resulting from testicular inoculation, while Levaditi and Yamanouchi (3) succeeded in producing infection by inoculation of the prepuce. With the introduction of skin inoculations, a decided advantage was gained in the study of experimental syphilis. Not only were the conditions of the inoculation more akin to the mode of infection in the human subject, but the lesions themselves were more analogous to the primary lesions of man. In addition, the infection took place in a region more accessible to observation and the reaction to inoculation could be followed directly, which in itself was a decided advantage.

Of the various skin areas in which inoculation was attempted, the scrotum proved to be best adapted and is now the only area used for purposes of routine inoculation. The methods devised for scrotal inoculation were of three types, all of which were quite simple and consisted in the implantation of bits of infected tissue beneath the skin of the scrotum, in the injection of a fluid medium containing the infecting organisms, or in scarification of a skin area and the local application of the virus. Satisfactory results have been reported from the use of all three of these methods.

EXPERIMENTAL.

In our own work upon scrotal infections of *Treponema pallidum*, the experimental conditions employed were much the same as those outlined in the case of the testicular infections (4).

Animals Used.—A great variety of animals was used, but those preferred were young albinos, grays, browns, or Dutch belts. Individual animals were chosen in which the scrotum was thin and delicate and as free from hair as possible. Old animals with thick, fibrous, or redundant scrota gave results inferior to those of younger animals with more active tissue.

Method of Inoculation.—Inoculation of the scrotum was carried out according to one of two methods. The procedure commonly employed was essentially the same as that described by Tomaszewski (5) which consists in the implantation of a small piece of infected tissue in a pouch prepared for it in the scrotum. The tissue used was either an infected testicle or a granulomatous skin nodule removed with as little contamination as possible and cut into pieces measuring from 3 to 5 mm. in diameter. The inoculation was performed either by the use of a trocar needle of 8 to 9 gauge inside diameter or by snipping the superficial layers of the scrotum with scissors and introducing the fragment of tissue by means of a pair of forceps; the latter method was in general preferred. The only precautions necessary were the observance of reasonable cleanliness and making the implants as superficial as possible.

Both of these methods proved highly satisfactory when rapid development of large skin lesions was desired. They are not adapted, however, to the study of the finer details of the reaction to infection, and for this purpose, we used an intracutaneous or subcutaneous injection of a virus emulsion prepared as for testicular inoculation except that the emulsion contained a greater number of spirochetes, averaging 5 to 10 to the microscopic field. In this operation, the scrotum was cleansed as usual and 0.1 to 0.2 cc. of the emulsion injected by inserting the needle well above the point to be inoculated and carrying it downward to the desired location.

Material Studied.—During the past 4 years, we have inoculated a large number of rabbits by these two methods. Among the earlier animals, there was an occasional one in which infection could not be established with absolute certainty, but all animals inoculated during the last 3 years have developed characteristic lesions from which *Treponema pallidum* could be recovered.

The material afforded by these rabbits was used in various ways. After well developed lesions had become established, a majority of them were used for therapeutic experiments and hence were not available for further study of the course of the local infection except where recurrent lesions developed. Some lesions were excised for histological study at various stages of their development, but a large number of rabbits was held under constant observation throughout the course of the local infection,—some of them as long as 18 months and a few for 2 years or longer.

Reaction Following Inoculation with a Virus Emulsion.

By whatever method scrotal inoculation is performed, the specific reaction which takes place is essentially the same. The early stages of the reaction can be traced more accurately, however, by the use of some methods than by others, and for this purpose, we used an emulsified virus which is but little more than a diluted tissue extract containing the *pallidum*. When inoculations are properly performed by this method, there is very little traumatism, and the slight inflammatory reaction which may develop on this account disappears completely within 24 to 48 hours. Thus, one is not only able to keep the dose of virus used comparatively uniform and to control the conditions of inoculation, but the resulting reaction is as little complicated by extraneous circumstances as is practicable upon any large scale. The chief objection to the method is that it tends to diffuse the infection over a wider area and the resulting reaction is not always so sharply circumscribed as one might wish.

Incubation Period.

The incubation period of scrotal infections produced by inoculation with a virus emulsion came within the comparatively narrow limits of 1 and 4 weeks. Under favorable circumstances, a definite reaction was usually recognized within 10 days to 2 weeks after inoculation and in many instances by the end of the 1st week. Occasionally, the specific reaction developed more slowly and characteristic lesions were not recognizable for several weeks.

The average incubation period of scrotal infections was somewhat shorter than that previously given for the development of gross or clinical alterations in the testicle but corresponded fairly well with

the time within which lesions could be demonstrated microscopically or within which multiplication of spirochetes could be determined by dark-field examination of fluid obtained from the testicles.

Form of the Initial Reaction.

The specific reaction in the scrotum appeared in one of three general forms differing somewhat according to the location of the lesion. The first and most important form of reaction began as small circumscribed swellings or diffusely spreading patches of a rose-pink color situated in the papillary and reticular layers of the skin. These lesions were soft or gelatinous in character and were frequently associated with a rich vascular network such as that shown in Fig. 1, which is probably the most significant feature of the syphilitic reaction. The second form taken by the initial lesion was that of a minute translucent nodule or plaque of induration likewise situated in the papillary layer of the skin but more superficial than the first (Fig. 5). The third type of lesion appeared as an opaque porcelain-white nodule or plaque of extreme hardness situated in the depths of the scrotum and was usually connected with the outer surface of the tunica vaginalis. The first two forms of reaction were the most common and usually the earliest to appear.

Development of the Primary Lesions, or Chancres.

The development of primary skin lesions, or chancres, from initial foci of reaction, such as those described, followed a course which may be represented in general by Figs. 1 to 4 and 5 to 8 which were from two animals of the same series. As indicated in these illustrations, the progress of the infection was marked by an extension of the reaction and the occurrence of certain transformations. The infection tended to spread diffusely through the scrotum, and as the reaction about the focus of infection increased, lesions were formed which assumed the character of circumscribed nodular elevations or of flattened, diffusely spreading patches.

In the development of the gelatinous type of lesion (Figs. 1 to 4), the swelling first subsided to a slight extent and the color changed to a more coppery tint. The lesions then became firmer, and as the

induration increased, changed to more opaque, porcelain-white masses. Purplish red spots and streaks of congestion and hemorrhage then appeared over the central portions of these lesions and with them yellowish gray or yellowish brown areas of necrosis which tended to spread and become covered with thin scales or crusts. Finally, the central area of the lesion became necrotic and sloughed away or was covered by a more or less continuous crust.

In the case of circumscribed lesions such as those in Figs. 1 to 4, these changes led to the formation of elevated masses with a necrotic or ulcerated center surrounded by a zone or collar of induration. If the lesions spread diffusely, as they not infrequently did, the resulting lesion was a thickened or indurated patch over which the areas of necrosis were more diffusely scattered; the crusts were usually imperfectly developed and loosely attached, and while ulceration was slight, weeping patches were formed here and there.

When the scrotal lesions first appeared as translucent nodules or patches of induration, development of the lesions followed a slightly different course (Figs. 5 to 8). The vascular reaction about such areas was relatively less than in the preceding case. As the lesions increased in size, the central portions became more dense and opaque, while the overlying skin became smooth and glistening. The covering epithelium was thinned out until small defects or superficial areas of necrosis appeared and were covered by thin scabs or crusts.

When these lesions assumed the form of circumscribed nodules (Figs. 5 to 8), the area of necrosis enlarged and deepened with the growth of the lesion and was covered by a thick crust or formed a depressed ulcer surrounded by a mass of indurated tissue. The more diffuse lesions of this class appeared as parchment-like thickenings in the skin which either remained intact and appeared translucent, smooth, and glistening throughout or were irregularly covered by loosely attached scales with raw or ulcerated areas here and there.¹

The third form taken by the initial lesion in the scrotum, that of an opaque white nodule or plaque of induration in the depths of the scrotum, usually followed a still different course of development.

¹ See section below on diffuse scrotal lesions.

As in the case of the lesions just described, the vascular reaction about these lesions was of minor degree. They developed rather slowly as a rule and grew or spread in the form of extremely hard masses over which the skin was freely movable. The plaques usually remained deep seated and spread along the surface of the tunica vaginalis, but the nodular lesions gradually extended towards the surface so that the overlying skin not infrequently became involved. Necrosis and ulceration then took place with the formation of lesions much like those which have been described. However, many of these deep seated nodules did not involve the overlying tissues to a sufficient extent to bring about necrosis and ulceration but remained throughout as circumscribed nodular lesions in the deeper tissues of the scrotum.

The subsequent course of the reaction to infections produced in this way was essentially the same as that of an infection produced by other methods of inoculation and may be followed out in connection with the infection produced by implantation.

Reaction Following Inoculation by Tissue Implantation.

While inoculation by the use of tissue implants possesses certain advantages, there may be some difficulties attending its use. Due to unequal distribution of spirochetes in infected tissues as well as to inequalities in the vitality of the organisms present in different portions of a lesion, inoculations carried out by implantation may not yield results which are as uniform as from the use of methods by which the dose of virus can be kept more nearly constant. It is possible, however, to overcome these difficulties to a large extent by the observance of certain precautions: first, the use of infected tissues at a relatively early stage of the infection or only at a period when the infection is actively progressing; second, the use of only such portions of a lesion as are actively growing at the time; and third, as a means of insuring an adequate dose of organisms when any doubt exists, the use of pieces of tissue as large as can conveniently be used without causing necrosis and sloughing of the surrounding tissues. Obviously, judgment in the use of these precautions can be acquired only by experience. A second difficulty in the

use of this method is the traumatism necessarily inflicted and the chances offered for the development of secondary infections. These need not prove serious, however, since reasonable care in the performance of inoculations will remove such difficulties to a considerable extent.

For certain purposes, implantation has advantages over other methods of inoculation which more than compensate for its disadvantages. When properly safeguarded, large, actively growing skin lesions can be obtained more quickly and with greater certainty by this method than by any other method of inoculation with which we are acquainted.

Incubation Period.

The exact incubation period of infections produced by scrotal implantation is difficult to fix since a non-specific foreign body reaction frequently overshadows the specific reaction. As nearly as can be determined, however, the incubation period in our series of animals fell within much the same limits as for inoculations made with emulsions. There was usually a definite specific reaction about the implant, and spirochetes could be obtained from the surrounding tissues within 10 days to 2 weeks after inoculation and were obtained as early as the 3rd day, but it was not certain that the organisms found were more than transient invaders. In a few instances, incubation was unusually prolonged after inoculations performed by this method, and no specific reaction could be recognized for several months—in one instance 6 months and 6 days. This unusual prolongation of the incubation period probably had no connection with the method of inoculation used except as a possible instance of the inoculation of a small number of organisms or of organisms of low vitality, and is merely cited to show the time which may elapse before any definite reaction can be detected.

Early Reaction.

The reaction which takes place in the scrotum following inoculation by implantation may be regarded as partly one of wound healing, partly a foreign body reaction, and partly specific in character. During the first 24 hours, the scrotum became slightly reddened and

edematous, but this acute inflammatory reaction rarely lasted beyond the 2nd or 3rd day. Then the process of organization became established; the swelling subsided, leaving the skin about the implant slightly reddened, smooth, and glistening. This condition persisted for upwards of 7 to 9 days without producing any considerable increase in the size of the nodule. In some instances, the skin over the implant became necrotic and was covered by a crust or sloughed away leaving a depressed ulcer surrounded by a zone of granulation tissue. If no specific reaction had developed by this time, the nodule began to shrink and soften while the skin became relaxed as the process of organization or healing subsided. If the tissues about the implant retained their appearance of activity beyond the 10 day period, and especially if the reaction appeared to be increasing, it was usually found that a specific reaction had commenced.

However, the specific reaction usually became apparent after the process of organization had abated to an appreciable extent and appeared almost as a renewal of this process. The differences which could be recognized were that there was less reddening in the specific reaction, the tissues were more translucent and more definitely indurated, and the reaction resulted in a rapid growth of the lesions. In many instances, there was no break in the continuity of the two processes and it was impossible to determine exactly when the specific reaction began.

Not infrequently, multiple foci of infection resulted from inoculation by this method. Thus, lesions developed independently about the implant at the point of incision and occasionally other discrete foci were scattered along the track of the sinus.

Course of the Scrotal Reaction and Development of Typical Scrotal Chancres.

In following out the successive stages in the scrotal reaction and the development of what may be called typical scrotal chancres, it may be assumed that the specific element in the reaction is the same in all essential respects whether inoculation is carried out by the use of a virus emulsion or by tissue implantation. The mechanical conditions of the reaction in the two cases are quite different, however.

The production of an infection by the introduction of a solid mass of tissue into the scrotum undoubtedly exercises a considerable influence in itself upon the character of the lesions produced and favors the development of large circumscribed lesions, but chancres produced in this way do not always follow the same course of development, and the resulting lesions themselves may be quite different. It is well, therefore, to consider the growth of these lesions according to the general character of the reaction.

Three characteristic examples of chancre development following the use of this mode of inoculation are given in Figs. 9 to 12, 13 to 16, and 17 to 20. The first group of photographs (Figs. 9 to 12) represents stages of chancre development where optimum conditions of virus, animals, and technique were combined. The animal shown was one of a series of ten rabbits in which the results were remarkably uniform. The first photograph (Fig. 9) taken 10 days after inoculation shows a well established specific reaction. There were some edema and congestion of the scrotum, and the skin over the implants had undergone necrosis with the formation of hard dry crusts which showed a line of demarcation at their periphery. Both implants were surrounded by a distinct but narrow zone of specific granulation, more prominent on the left than on the right. These lesions developed very rapidly and by the 13th day (Fig. 10) presented all the characteristics of typical chancres with depressed ulcers surrounded by broad zones of induration.

With the growth of the lesions as illustrated in Figs. 11 and 12, taken 3 and 5 weeks respectively after inoculation, the diffuse congestion and edema of the scrotum persisted; the scrotum gradually became thickened and a few petechial hemorrhages appeared towards the lower end of the right scrotum (Fig. 11), which formed the center about which a second circumscribed lesion soon developed. Eventually, the entire scrotum of both testicles became involved in the specific reaction and the condition existing at the end of the 5th week was that shown in Fig. 12. On the right, there were two circumscribed lesions connected by areas of more diffuse infiltration, while on the left, there was a single massive chancre surrounded by tissues which were themselves markedly infiltrated and covered by diffusely spreading patches of hemorrhage and necrosis.

This is an instance of a high grade infection in the scrotum of the rabbit such as may be produced under favorable conditions and has been observed many times. It may not be out of place to point out that this case illustrates, in particular, first the extreme rapidity with which the infection may become established and typical circumscribed lesions developed, and secondly the inherent tendency of

pallidum infections to spread beyond the local confines of the initial focus of infection, together with something of the character of the reaction which occurs under such circumstances.

The second case in this group (Figs. 13 to 16) illustrates a course of chancre development which is more nearly an average than that just described. The reaction exhibited by this animal was of the same general character as that of the first, but differed in two essential respects. There was early ulceration of the lesions as in the former case, and by the 14th day, characteristic chancres with well defined collars of induration were present (Fig. 13). The growth of the lesions then ceased, the edema in the scrotum subsided, and the chancres themselves decreased in size (Fig. 14). During the 4th week, there was a slight recurrence of the edema (Fig. 15) and renewed growth of the circumscribed lesions which continued with slight remissions until large indurated lesions were formed (Fig. 16, 57 days after inoculation).

These two examples will serve to illustrate the type of reaction which takes place in most instances of active chancre development. A third form of reaction commonly seen is that illustrated in Figs. 17 to 20. This animal was one with a rather thick fibrous scrotum (note the scrotum in Figs. 17 and 18) and the implants could not be made so superficially as one would wish. The incubation period was somewhat longer, and the lesions did not begin to grow actively until about the end of the 4th week after inoculation. The photograph reproduced in Fig. 17 was taken at the end of the 5th week (34 days) when the nodules measured approximately 1 cm. in diameter. The skin about the nodules was still but little affected and for the most part retained its normal appearance. During the next 18 days, these lesions underwent a considerable transformation (Figs. 18 to 20); they increased in size and became more indurated, while the process extended to the overlying skin which became smooth, translucent, and highly refractile. Areas of necrosis then appeared upon the surface, and as these extended and contracted, the lesions flattened out into the form seen in Fig. 20. Specific reactions of this type were especially apt to occur when the lesions developed in the depths of the scrotum or when, for any reason, the reaction pursued a less active course and involvement of the skin with consequent necrosis and ulceration took place at a relatively late period in the growth of the lesions.

Multiple Chancres.—Multiple lesions of a chancre-like character developed in the scrotum under two different conditions, first as a result of simultaneous infection of several points in the scrotum, and second as a result of spread of the infection from a given focus. While lesions of both classes might be regarded as chancres, the significance of the two cases is different, and in the present connection, we shall refer only to lesions of the first class.

Inoculation of the scrotum of the rabbit by the methods described usually resulted in the production of unicentric lesions. Not infrequently, however, lesions appeared simultaneously at other points reached directly in the process of inoculation. The most common seat of the accessory chancre was the point of incision in the skin, and a typical instance of an early lesion of this kind is shown in Fig. 21, the photograph of which was taken 14 days after inoculation. Accessory lesions of this type were frequently abortive or were overgrown by the more vigorous reaction about the main focus of infection as was the case in this animal (Fig. 22, 36 days after inoculation).

A second case of a similar character is shown in Fig. 23. Originally there was an accessory focus of reaction on both sides of this animal. That in the right scrotum was abortive and had almost disappeared when this photograph was taken (29 days after inoculation), while the one on the left was growing quite actively.

In some animals the accessory chancres reach a considerable size even though they are encroached upon by the main chancre mass. Figs. 24 and 25 show a case of multiple chancres in which there were three foci of reaction in the right scrotum and two in the left (Fig. 24, 29 days after inoculation). As these lesions developed, the middle focus of infection on the right became obliterated, but the other accessory chancre grew almost as vigorously as the main lesion. On the left, the two lesions fused at their proximal borders forming a figure of eight chancre (Fig. 25, 49 days after inoculation).

In rare instances, one finds little difference in the vigor displayed by the several lesions present, and all may develop at about an equal rate as shown in Fig. 26 (47 days after inoculation). In addition to the group of multiple lesions described, all of which were situated in the skin itself or pointed upon the skin surface, there was another interesting group of multiple focal lesions in which the foci of reaction were located at different levels in the scrotum—one above the other; these lesions were situated in the skin and upon the outer surface of the tunica vaginalis. This form of localization occurred especially after subcutaneous inoculation with a virus emulsion and is of especial interest in revealing a tendency of *Treponema pallidum* to localize in these two structures.

These few examples of the reaction to infection in the scrotum and the growth of the primary lesions will serve to illustrate what may be called typical skin reactions and typical chancre development in the sense that they represent processes which are in themselves perfectly characteristic and result in the formation of lesions which are easily recognized as lesions of a specific character. In many instances, however, reactions occur and lesions develop which are not so well recognized as processes of a syphilitic nature, and we may refer briefly to a few instances of this kind.

Influence of Different Elements of the Specific Reaction upon Chancre Development.

Irregularities of Chancre Development.

The condition which gives the characteristic picture to the specific reaction in the skin and to the chancre itself is the maintenance of a certain balance among the several processes which take part in this reaction. It was found that when for any reason this balance was disturbed, the character of the reaction was altered and the lesions became modified in accordance with existing conditions. The irregularities of chancre development which were most commonly seen were associated with the occurrence of excessive edema or of congestion and edema, with conditions simulating an acute inflammatory reaction, or with irregularities in the process of granulation, the cause of which cannot be discussed.

Edema.—It was found that excessive edema with or without congestion might occur at any time during the course of the local infection. When it developed at an early stage of the infection, it not infrequently overshadowed the proliferative reaction and produced a lesion which showed chiefly a depressed ulcer with a firm margin surrounded by an edematous skin (Fig. 27). In some instances, this condition persisted for some time, while in others it was no more than an important feature of the reaction or an event in the course of the reaction. Figs. 28 and 29 illustrate an instance of this kind in which the specific reaction was proceeding rather slowly, and at the end of the 3rd week, there were ulcers surrounded by a narrow zone of induration. An acute edema then developed (Fig. 28), and with the appearance of this edema, the focal lesions began to grow at an extremely rapid rate; in 2 weeks, they had reached the condition shown in Fig. 29, the edema persisting to an appreciable extent all the while.

Late edema or edema occurring after characteristic lesions had developed was also seen occasionally and was so marked in some instances as to obscure completely the character of the lesion (Fig. 30).

Acute Inflammatory Reactions.—A small number of rabbits in the series showed an early reaction resembling an acute inflammatory process. There was the usual inflammatory reaction during the first few days following inoculation, and as this subsided, an extremely active process of granulation set in; the tissues surrounding the implant remained reddened or cyanotic and were quite firm, while the zone of induration increased rapidly. The center of the nodule became necrotic and sloughed, leaving a moist, depressed ulcer, or, if no slough occurred, the necrotic tissue softened to a creamy semifluid mass covered by a crust. As the reaction progressed, the tissues surrounding the zone of granulation or even

the entire scrotum became congested and edematous (Fig. 31). This condition usually lasted for not more than 3 to 5 weeks before regression set in. The lesions which appeared subsequently were usually of a minor character, but in some instances, typical chancres were formed as in the case illustrated (Figs. 31 and 32).

At first we were inclined to regard these acute reactions as non-specific, inflammatory processes, but investigation showed that this was not entirely correct. Spirochetes were present in considerable numbers both in the zone of granulation and in the edematous portions of the scrotum entirely removed from the region of the implant, but secondary infection could not be excluded as a factor in the reaction. However, true suppuration did not occur as it usually did where pyogenic organisms were present to any considerable extent. It was noted that these reactions were more frequent when large implants were made from testicles during an early stage of extremely active infections. It seemed possible, therefore, that these were cases of acute specific reaction influenced to a greater or less extent by bacterial infection as well as by the implant itself.²

Irregularities in the Process of Granulation.—In many instances, irregularities in the development of scrotal chancres were traceable to peculiarities in the process of granulation, and since this is such a noticeable feature of what we are accustomed to regard as the typical *pallidum* reaction, the peculiarities naturally took the form of a lowered intensity of reaction or of some irregularity in the process, both of which were commonly seen in scrotal infections in the rabbit.

The case illustrated in Figs. 33 to 36 furnishes an example of an abortive skin reaction with subsequent development of granulomatous nodules in the subcutaneous tissues of the scrotum. The lesions first formed were simple ulcers surrounded by a thin, parchment-like zone of induration in which spirochetes were present (Fig. 33, 35 days after inoculation). On the left, the zone of induration increased slowly and a characteristic skin chancre was produced (Fig. 34, 83 days after inoculation). Meanwhile, the skin lesion on the right had regressed, and a small nodule had developed in the subcutaneous tissues (Fig. 34). Then followed a period during which the lesion on the left extended into the subcutaneous tissues, while the induration in the skin diminished and the ulcer healed (Figs. 35 and 36, 139 and 188 days after inoculation).

² In this connection, it may not be out of place to call attention to the fact that in all syphilitic infections, the presence of a toxin as an exciting factor distinct from the organisms must be kept in mind. We have avoided introducing this element into the discussions, since we are not prepared, as yet, to make any definite statement upon this point. It seems not unlikely, however, that certain features of the syphilitic reaction may find their explanation in the action of such a toxin, and this applies to reactions of the type just described.

A second case of even less characteristic chancre formation is shown in Figs. 37 to 40. The initial skin lesion in this animal was no more than a thickened patch, the surface of which was smooth and glistening with small erosions here and there; some of these were covered by scales, while others were of a weeping character. These lesions underwent many transformations (Figs. 37 to 40), but never developed beyond the point shown in Fig. 37 which represents the condition existing 36 days after inoculation. Here again, the granulomatous lesions which ultimately developed were subcutaneous in origin and were of very slow and irregular growth (Figs. 38, 39, and 40; 92, 106, and 127 days after inoculation).

Diffuse Scrotal Reactions and Transformations of Diffuse Lesions.

As we have already pointed out, there is a tendency on the part of *pallidum* infections to spread beyond the point of inoculation, and not infrequently this local extension of the infection gives rise to lesions of a more diffuse character than those which have been described. No sharp line of distinction could be drawn between these two groups of lesions; it appeared that one was but a step removed from the other, and diffuse and circumscribed processes were frequently coexistent, or one type of lesion might be transformed into the other, instances of which have already been noted.

As a connecting link between these two forms of scrotal reaction, attention may be called to the lesions shown in Figs. 41 to 44. The photograph reproduced in Fig. 41 was taken 33 days after inoculation. In this animal, there was a moderate but fairly well defined thickening in the skin about the incisions as well as about the implants, and the characteristic feature of the lesions was the spreading necrosis in the skin over the implants and the formation of loosely attached scales, or exfoliation, over all affected parts of the skin, which is shown very well in the left scrotum (Fig. 41).

The second photograph (Fig. 42) shows a later stage of a similar lesion. In this animal, there are two things to be noted, first the puckered scar-like areas on both sides, and second the peculiar appearance of the skin about these areas. The skin was in general of a parchment-like character, very smooth and translucent with grayish yellow or yellowish brown scales or crusts distributed over its surface. Needless to say, these portions of the scrotum contained spirochetes in abundance.

The other two figures on this plate (Figs. 43 and 44) illustrate a somewhat different manifestation of the syphilitic infection. In Fig. 43, there is shown a rather irregular and slightly nodular thickening in the right scrotum with a scurfy condition of the skin over portions of the lesion; on the left, there is a puckered ulcer with moderate thickening of the surrounding skin. These were the lesions as

they appeared 76 days after inoculation. Subsequently the thickening or induration in the skin became diminished, but instead of healing, the lesions spread in an irregular way, producing a peculiar serpiginous necrosis and ulceration over a considerable part of the scrotum (Fig. 44, 118 days after inoculation). There was no suggestion of an acute inflammatory process associated with these changes, and spirochetes were present in fluid drawn from about the lesions.

The next series of photographs (Figs. 45 to 48) shows successive stages in the transformation of a diffuse scrotal infection which reverses somewhat the sequence of events illustrated in the preceding series. The reaction began here as a diffuse infiltration with exfoliation of the epithelial covering of the scrotum (Fig. 45, 53 days after inoculation). After a time, the scaling, or exfoliation, ceased, and the skin became very smooth and translucent. At the same time, the skin became diffusely indurated, and circumscribed nodules appeared in the subcutaneous tissues on both sides. These nodules grew rather slowly, and on the 151st day after inoculation, the condition presented was that shown in Fig. 46. The subcutaneous nodule in the left scrotum was later excised, but the infection in the scrotum persisted in spite of the operation. The nodule on the right continued to grow, and as it developed, the infiltration in the skin diminished. There was, however, a slight recurrence of the exfoliative reaction (Figs. 47 and 48, 165 and 172 days after inoculation).

No attempt can be made to indicate the diversity of conditions to which the specific reaction in the scrotum may lead. By comparing and following the various phases and stages of the reaction, it will be seen that the elements in these reactions are the same in all cases; all show a vascular reaction, a certain amount of exudation, infiltration, and proliferation together with secondary necrosis and ulceration or exfoliation where the necrosis is superficial. The lesions we regard as but an expression of the operation of these factors in the reaction and the balance obtaining in a given case at a given time. As the response to the specific infection varies in different animals or even in the same animal from time to time, so do the lesions, and the possibilities of such variations are almost infinite. The consideration of this phase of the local infection will be taken up in the second part of this paper.

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EXPLANATION OF PLATES.

The first four illustrations are drawings made from the living animal and are based upon photographs taken at the time. The remaining illustrations are reproductions of untouched photographs. All objects are represented at their natural size. The statements of time refer in all cases to the time after inoculation unless otherwise stated.

PLATE 72.

FIGS. 1 to 4. Successive stages of the reaction to infection in the scrotum following inoculation with a virus emulsion.

FIG. 1. 2 weeks. Localized swelling in the scrotum with vascularization of the area. An unusually characteristic reaction.

FIG. 2. 23 days. Early induration with congestion and petechial hemorrhage from the vessels in the center of the lesions.

FIG. 3. 28 days. Anemic necrosis and exfoliation.

FIG. 4. 41 days. Circumscribed and indurated chancres with marked central necrosis and ulceration.

PLATE 73.

FIGS. 5 to 8. Development of scrotal chancres from focal infiltrations following inoculation with a virus emulsion.

FIG. 5. 1 week. Minute focus of induration in the right scrotum and diffuse infiltration with vascularization in the left.

FIG. 6. 19 days. Circumscribed areas of infiltration and induration. The skin over the center is becoming thin and translucent; slight congestion and hemorrhage on the right.

FIG. 7. 28 days. Surface necrosis and exfoliation.

FIG. 8. 43 days. Chancres with well developed collars of induration and marked central necrosis and ulceration.

PLATE 74.

FIGS. 9 to 12. An intense reaction to infection with the development of circumscribed indurated chancres and subsequent extension of the lesions following implantation in the scrotum.

FIG. 9. 10 days. The early syphilitic reaction following inoculation by implantation. There are diffuse edema and congestion of the scrotum and the implants are surrounded by a narrow zone of induration, while the skin over their center has been converted into hard dry crusts.

FIG. 10. 13 days. The early chancre. Note especially the well marked collars of induration and the tendency to exfoliation in the right scrotum.

FIG. 11. 22 days. The diffuse reaction has persisted and the edema has given place to infiltration and thickening of the scrotum.

FIG. 12. 36 days. Large indurated chancres. Diffuse extension of the reaction with widespread necrosis and ulceration of the scrotum.

PLATE 75.

FIGS. 13 to 16. A usual case of scrotal reaction showing cyclic changes in the development of the lesions. Scrotal implantation.

FIG. 13. 14 days. Typical circumscribed lesions with depressed ulcers; edema and congestion of the scrotum.

FIG. 14. 21 days. The lesions have decreased in size; edema and congestion have subsided.

FIG. 15. 28 days. Renewed growth of the chancres and reappearance of scrotal edema.

FIG. 16. 52 days. Large indurated chancres with well marked central necrosis and the formation of ulcers.

PLATE 76.

FIGS. 17 to 20. Deep seated scrotal infection, characterized by slow growth of the lesions and late involvement of the skin. Implantation.

FIG. 17. 34 days. An early stage of the reaction. Skin but slightly involved.

FIG. 18. 41 days. Involvement of the skin with obliteration of papillæ and beginning skin necrosis.

FIG. 19. 44 days. A very active advance of the process indicated in Fig. 18.

FIG. 20. 52 days. The reaction is still quite active. The lesions are extending laterally and the central necrosis is increasing.

PLATE 77.

FIGS. 21 to 26. Multiple foci of primary reaction. Multiple chancres. Implantation.

FIG. 21. 14 days. A well marked accessory focus of reaction at the point of incision of the scrotum on the right.

FIG. 22. 36 days. Overgrowth of the accessory chancre by the main focus of reaction.

FIG. 23. 29 days. Quadruple foci of reaction. The accessory lesion on the right is already regressing.

FIG. 24. 29 days. Triple and double foci of reaction.

FIG. 25. 49 days. Subsequent development of chancres from four of the five initial foci of reaction in Fig. 24.

FIG. 26. 47 days. Quadruple chancres. An unusual case of equal reaction about all of four centers of reaction.

PLATE 78.

FIGS. 27 to 32. Variations and irregularities of the specific reaction. Edema, congestion, and acute inflammatory reactions.

FIG. 27. 16 days. Early circumscribed chancres with diffuse congestion and marked edema of the scrotum. Spirochetes recovered from the point indicated by the arrow on the right.

FIG. 28. 25 days. Focal lesions with acute edema and congestion of the scrotum.

FIG. 29. 39 days. Marked growth of the focal lesions following the exudative reaction in the scrotum.

FIG. 30. 102 days. Sudden development of edema in the scrotum which was the seat of an old indurated lesion. (Compare left.)

FIG. 31. 11 days. An intense granulomatous reaction associated with edema and marked congestion of the scrotum, the exact nature of which is uncertain (see text).

FIG. 32. 50 days. The same animal as in Fig. 31, showing the development of typical indurated chancres following partial regression of the previous lesions.

PLATE 79.

FIGS. 33 to 36. Successive stages of an atypical granulomatous reaction.

FIG. 33. 35 days. Simple ulcers of the scrotum with slight induration about their margins and base.

FIG. 34. 83 days. Slow but characteristic development of the lesion in the left scrotum. Regression of the lesion on the right with the formation of a small subcutaneous nodule underneath the original lesion.

FIG. 35. 139 days. Continued growth of the lesion on the left while that on the right has diminished in size.

FIG. 36. 188 days. Complete healing of the original skin lesions with the development of active subcutaneous nodules on both sides.

PLATE 80.

FIGS. 37 to 40. Successive stages of an irregular scrotal reaction.

FIG. 37. 36 days. Patches of superficial induration in the skin with necrosis and exfoliation.

FIG. 38. 92 days. The patch of induration on the left is still present, while that on the right has almost completely resolved. Subcutaneous nodules forming on both sides.

FIG. 39. 106 days. On the right, there is a small patch of glassy induration in the skin and beneath this a small indurated nodule surrounded by a mass of diffusely thickened tissue. On the left, the subcutaneous nodule is developing rapidly.

FIG. 40. 127 days. Active development of both nodules with extension to the skin. Note the recurrence of the indurated patch in the right scrotum.

PLATE 81.

FIGS. 41 to 44. Diffuse syphilitic processes following scrotal inoculation.

FIG. 41. 33 days. Slight granulomatous reactions about both the implants and the points of incision in the scrotum. The characteristic feature of the reaction is the spreading necrosis and exfoliation over the areas involved.

FIG. 42. 105 days. Diffuse infiltration about the site of inoculation with superficial necrosis and exfoliation. No typical chancre was ever formed in this animal.

FIG. 43. 76 days. A group of irregular and slightly nodular lesions in the right scrotum with surface necrosis and slight exfoliation. On the left, there is a diffuse infiltration of the scrotum with necrosis over the central area.

FIG. 44. 118 days. Same animal. Spreading serpiginous necrosis involving the areas of infiltration.

PLATE 82.

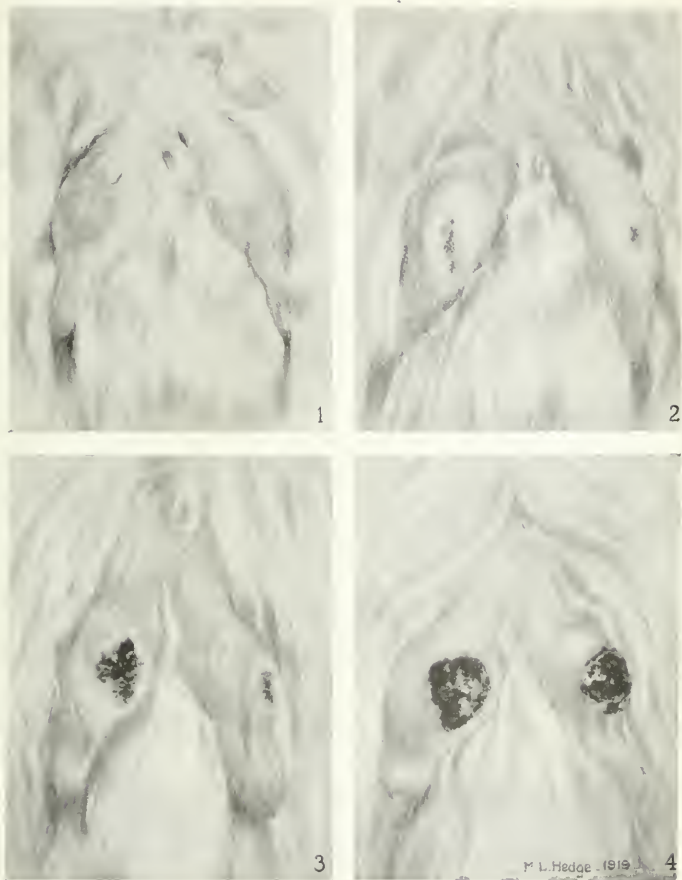
FIGS. 45 to 48. Successive stages in the transformation of diffuse scrotal lesions.

FIG. 45. 53 days. Diffuse infiltration of the scrotum, slight necrosis, and well marked exfoliation of surface epithelium—the initial lesion.

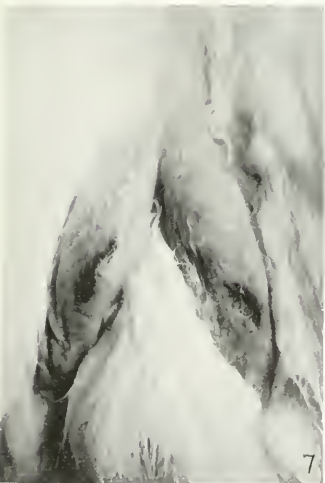
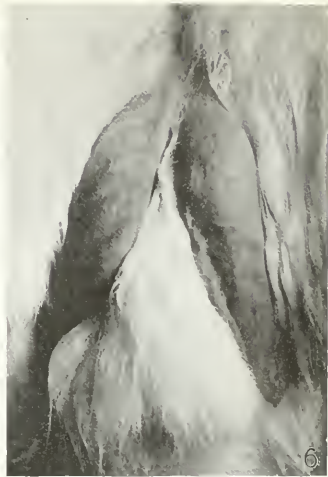
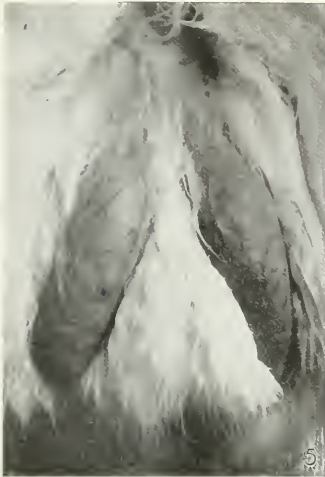
FIG. 46. 151 days. Marked diffuse infiltration of the skin with obliteration of the papillæ; large granulomatous nodules developing in the subcutaneous tissues.

FIG. 47. 165 days. Subcutaneous nodule removed on the left; that on the right still increasing, while the infiltration of the scrotum is slightly diminished.

FIG. 48. 172 days. The operative wound is practically healed; active infection still persists in the skin on the left; the nodule in the subcutaneous tissues is increasing, but the infiltration of the skin has almost disappeared; there is slight desquamation of surface epithelium.



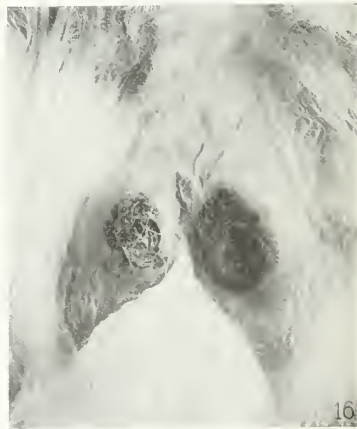
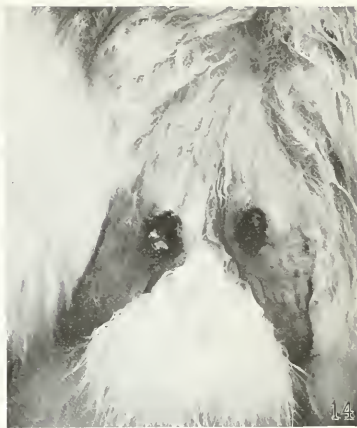
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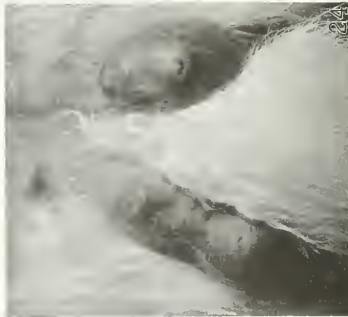
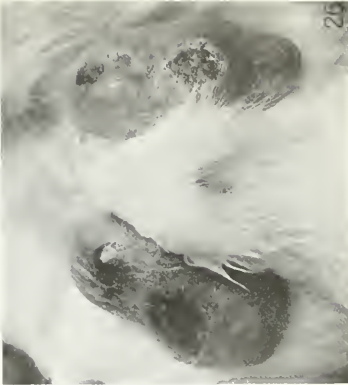
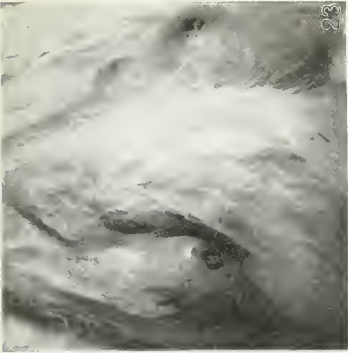
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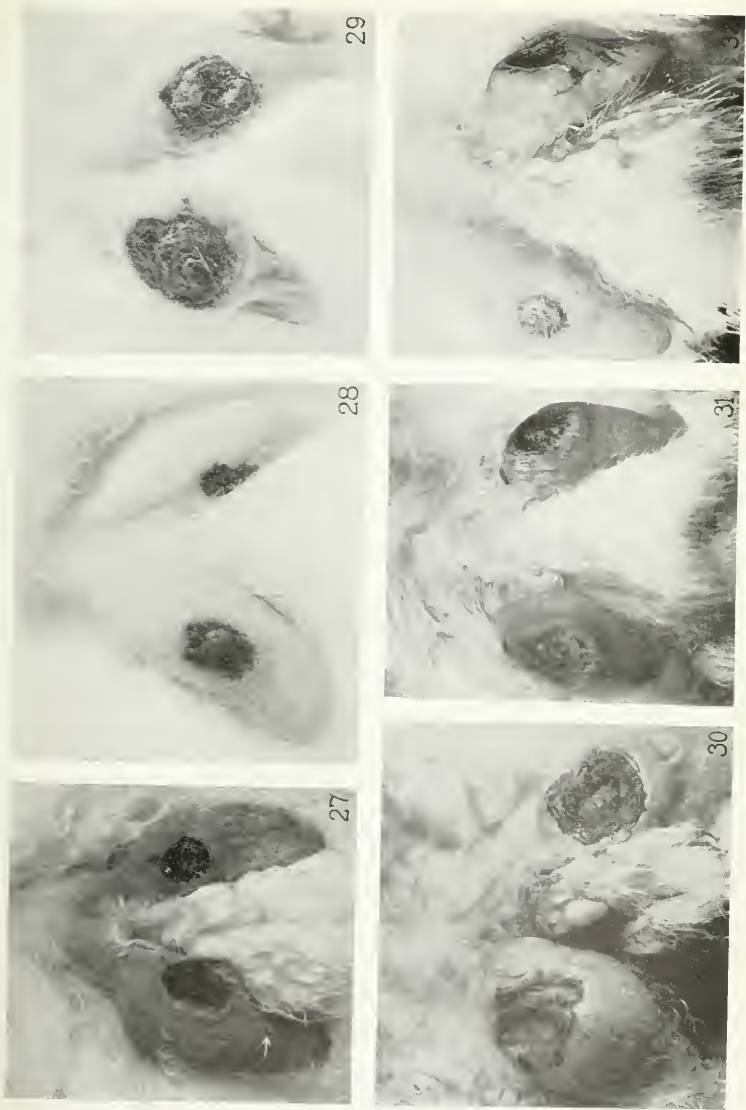


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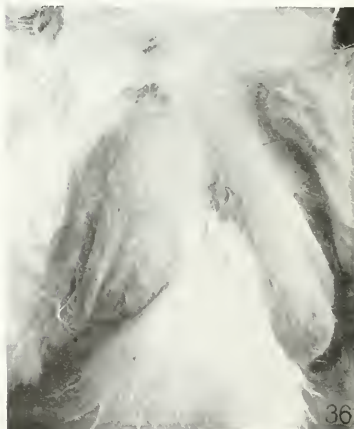
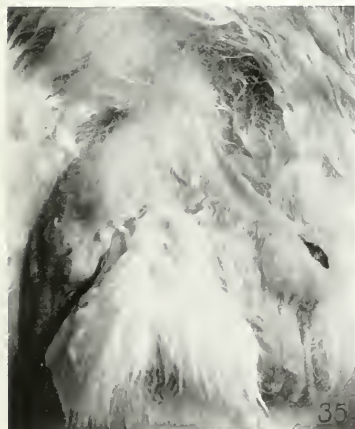


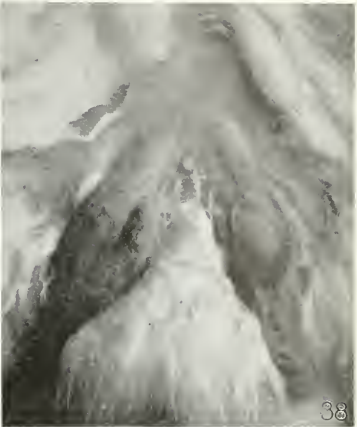
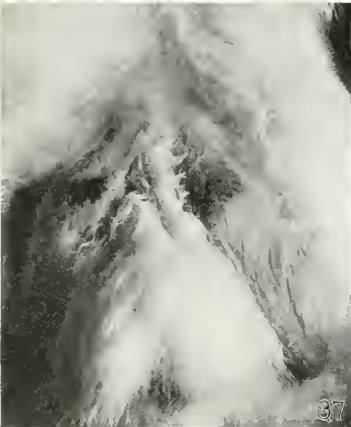




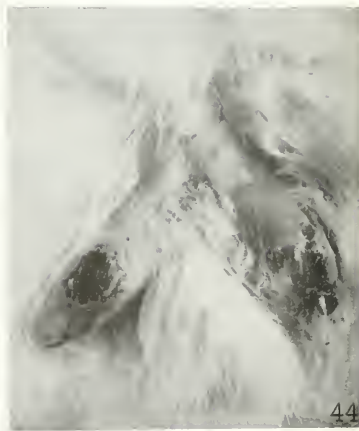
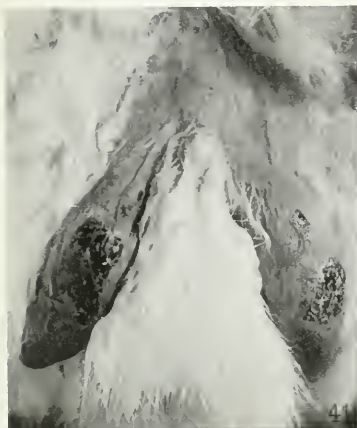


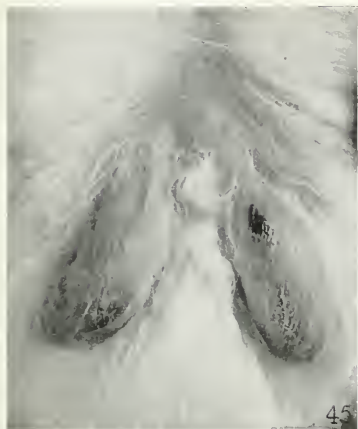
(Brown and Pearce: Experimental syphilis in the rabbit. 11.)





(Brown and Pearce: Experimental syphilis in the rabbit. 11.)





EXPERIMENTAL SYPHILIS IN THE RABBIT.

II. PRIMARY INFECTION IN THE SCROTUM.

PART 2. SCROTAL LESIONS AND THE CHARACTER OF THE SCROTAL INFECTION.

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PLATES 83 TO 89.

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In Part 1 of this series, the subject of scrotal syphilis in the rabbit was considered from the standpoint of the local reaction to infection. The various elements in this reaction were noted, and the process was traced from the time of inoculation to the development of characteristic skin lesions. Especial attention was called to the multiform character of these reactions and the influence of various elements in the reaction upon the character of the lesion produced, and while cause and effect were not entirely dissociated, the main emphasis was placed upon the process, and no detailed description of the resulting lesions was attempted. This phase of the subject is, however, of great importance in itself, and a more comprehensive view of the scrotal lesions is essential to a correct understanding and use of the experimental infection.

Primary Lesions of the Scrotum.

A classification of scrotal lesions which would be acceptable from all standpoints is impossible, but in order to simplify the description of these lesions, they may be separated into several general groups based partly upon fundamental differences in growth tendencies and partly upon structural differences in the lesions themselves, neither of which represent sharply defined or entirely fixed characteristics.

The first division to be made is one which has already been suggested, that of circumscribed and diffuse lesions. The circumscribed chancres include the lesions in which growth takes place from a central focus and tends to be more or less equal in all directions, leading to the development of raised or elevated masses of a circumscribed and indurated character. The diffuse chancres, on the other hand, include lesions which tend to spread laterally and assume a more or less flattened condition. In our experience, chancres of the first group were by far the more common of the two, and since they are more analogous to the classical primary lesions seen in man, they might also be spoken of as typical chancres in contradistinction to the less well defined lesions of a diffuse character.

The variety of primary lesions and the differences which exist between extreme examples of these two groups of lesions are so great that without the intervening links they could hardly be recognized as results of one and the same pathological process. Considerable care has been devoted, therefore, to the mere matter of selection and arrangement of illustrations with a view to giving as accurate an impression of scrotal chancres as possible, both as regards the character of the lesions and the relationship existing between lesions of different types.

Circumscribed or Typical Scrotal Chancres.

The circumscribed, indurated lesions of the scrotum studied by us were lesions which as a class tended to grow to a very large size—much larger than the primary lesions commonly seen in man. These lesions differed according as they arose from one part of the scrotum or another and according to the mode and rate of their development. Based upon these differences, the typical chancres of the scrotum might be divided into two main groups, the nodular and the lenticular or discoid chancres.

Nodular Chancres.—The nodular chancres comprise a large group of lesions whose chief characteristics were an irregular spherical form, a relatively small and sharply circumscribed area of superficial necrosis and ulceration, or the absence of any ulceration, and extreme induration. This general class of chancres may be separated into two

divisions, the ulcerated and the unulcerated chancres, characteristic examples of which are given in Figs. 1 to 6 and 7 to 12.

The nodular chancres appeared to develop from the subpapillary layers of the skin, the subcutaneous tissues, or the outer surface of the tunica vaginalis. They began their existence as more or less spherical masses of induration situated in the deeper portions of the scrotum. The growth of the lesions was comparatively rapid, while necrosis took place more slowly and was of relatively slight extent, at least during the period of active development of the lesion. As the overlying skin became involved, therefore, the area of necrosis was small and a sharply circumscribed depressed ulcer was formed, the margins of which were rounded and intensely indurated (Figs. 1 and 2). These lesions were of a dense fibrous structure, and the small area of central necrosis was usually surrounded by a thick wall of well vascularized living tissue.

The chief variations among the chancres of this class appeared to have their origin in the balance existing between the phenomena of growth and necrosis. The typical condition was that shown in Fig. 1. In a second group of cases (Figs. 2 and 3), growth and necrosis were more nearly balanced and a more extensive destruction of the central portion of the lesion took place. This condition, however, appeared to be referable to a corresponding variation in the structure of the surrounding tissue which in these cases was unusually dense or of almost ivory-like hardness. This condition is suggested in Fig. 2.

The chancres reproduced in Figs. 4 and 5 illustrate another modification commonly seen in chancres of this group occurring either during the period of active growth or as a terminal alteration. The condition was no more than a spreading necrosis which in actively growing lesions produced a unilateral flattening due to cessation of growth in the direction of the skin surface. This alteration was especially noticeable with large and rapidly growing lesions such as those in Figs. 4 and 5. It was a common occurrence, however, with all classes of chancres after active growth had slackened or ceased. In these cases, extension of the necrosis due to a continuation of secondary alterations in the granulomatous tissue resulted in a gradual destruction of the blood vessels and shutting off of the blood supply to the chancre.

This secondary necrosis took place in one of two ways which may be suggested by a comparison of the right and left chancres in Figs. 5 and 6. With large lesions showing a unilateral flattening with surface necrosis, there was a tendency for the spreading necrosis to surround the lesion from the outside (Fig. 5), while in lesions with central necrosis and depressed ulcers, the extension of the necrosis took place radially. This latter type of change, as illustrated in Fig. 6, was, of course, not confined to terminal alterations in chancres but occasionally was seen as an early feature of the lesion and is entirely analogous to the condition shown in Fig. 3.

The second division of nodular chancres differed from the first chiefly in respect to necrosis and ulceration. The two groups of lesions had a common origin and a common structure; in one group, surface necrosis and ulceration of moderate degree were characteristic features of the lesions, while in the other, these secondary alterations were of very limited extent or entirely absent. The photographs reproduced in Figs. 7 to 12 have been arranged to show successive gradations in the tendency to skin involvement and ulceration.

It may be of interest to note that all the chancres used for illustration in Figs. 1 to 12 were produced by a common organism—the Zinsser-Hopkins strain of *Treponema pallidum*. Formerly, lesions of the second type were the more common of the two, while at present the order of frequency is reversed.

Lenticular and Discoid Chancres.—The lenticular and discoid chancres which form the second main division of typical scrotal chancres differed from the first or nodular chancres in being lesions of a more flattened character, in the occurrence of a more widespread necrosis, and in possessing a lesser degree of induration as a rule. Typical examples of lesions of this class are given in Figs. 13 to 24.

The lenticular and discoid chancres appeared to arise mainly from the papillary layers of the skin, and the chief direction of their growth was in a plane parallel with the skin surface. Some of these chancres showed the thicker center and sloping edges of the lenticular lesion, but more commonly the edges were elevated fully as much or even more than the center, giving to the lesions a discoid rather than a lenticular form.

The growth of these chancres was extremely rapid, as a rule, and was associated with widespread skin involvement. Early in the course of their development, the skin over the central portion of the lesion became necrotic, and the extension of this zone of necrosis tended to keep pace with the growth of the lesion, spreading over the surface of the lesion as well as through its deeper parts. In some instances, the necrotic area softened and sloughed away with the formation of a true ulcer, but in others, the necrotic tissue remained firmly attached to the underlying structures, forming a thick imbricated crust (see figures).

Outside the zone of necrosis, the skin covering these chancres was markedly infiltrated and presented an unusually smooth and translucent appearance—a condition which not infrequently extended entirely to the outer edges of the lesions.

These chancres were as a class less indurated than the nodular chancres. In exceptional instances, however, they showed the same dense, fibrous structure and intense induration as nodular chancres (see Fig. 27), but more commonly they were of a mucoid or cellular character, and while these lesions were indurated, their induration was of an elastic quality, giving the impression of tension rather than of hardness.

Rapid growth and widespread necrosis were such striking characteristics of this group of chancres that two sets of illustrations are given to show the extremes to which these processes may go. Figs. 19 to 21 represent chancres produced by inoculation with a virus emulsion. The original lesions in this animal were excised 20 days after inoculation. There was a prompt recurrence, however, and Fig. 19 shows the lesions present 45 days after inoculation, or 25 days after excision of the lesions. From this point on the evolution of these chancres was extremely rapid. Within 20 days, they increased to the size shown in Fig. 20; during the next 3 weeks, growth continued at a somewhat slower rate with a deepening and extension of the necrosis (Fig. 21). The chancre on the left appears here much smaller than that on the right but was in reality almost as large. The appearance is due to foreshortening as only one chancre could be brought squarely in front of the camera.

The other three figures (Figs. 22 to 24) are from chancres produced by implantation. They show an equally rapid growth with the production of lesions which in this case almost lapped the testicles. The necrosis in these chancres was of the type of a dry gangrene spreading diffusely over the surface without a slough and forming heavy crusts composed of concentric layers and rings of ne-

crotic tissue. The figures represent periods of 39, 46, and 60 days respectively after inoculation.

The entire group of lenticular and discolored chancres thus far presented from Figs. 13 to 24 were produced by the Nichols strain of *Treponema pallidum* and show a certain degree of uniformity in type. Some of the more important variations of this class of chancres are indicated in Figs. 25 to 30.

The first two chancres of this group (Figs. 25 and 26) are of a decidedly mucoid character and the skin over the lesions is quite smooth and translucent. One of these chancres is flat while the other shows a tendency towards a more nodular form. In contrast to these lesions which again were products of the Nichols' organism, the two chancres in Figs. 27 and 28 present a decidedly different appearance. They were older lesions but represented a state of development comparable to that of the other two lesions. These chancres were of a dense fibrous character and were intensely indurated. They were produced by the Zinsser-Hopkins strain of *Treponema pallidum*.

The two remaining photographs of this group (Figs. 29 and 30) are given to illustrate chancres of an indolent type or ones which are less vigorous than most of those previously shown. The first of these chancres showed a normal rate of growth but an irregular growth; the lesions were only moderately indurated and there was but slight skin involvement apart from the area of necrosis. The second set of chancres (Fig. 30) showed a very slow and irregular development, with an undermining necrosis. The skin about the lesions was relaxed and there was the merest shell of living and growing tissue. Lesions such as those in Figs. 29 and 30 are more or less constantly at the border-line of regression and their growth may be interrupted by the slightest of causes.

The chancres thus far described will serve to give a fair impression of the principal chancre types and of the scrotal chancre at its highest state of development. The modifications of these types were so numerous that it would be futile to attempt to describe such a series of lesions. As we pass from these more typical chancres, we come to a group of lesions showing a progressive loss of the characteristics by which we are accustomed to identify primary skin lesions and eventually to lesions which are quite atypical in character. The photographs reproduced in Figs. 31 to 36 are intended to indicate this transition from the typical circumscribed chancres to lesions of a more diffuse and less typical character.

The first three photographs (Figs. 31 to 33) show lesions with all the characteristics of active skin lesions. The chancres in Fig. 31 were virtually thickened plaques with wide shallow ulcers, but the narrow margins as well as the base were markedly indurated. The chancres in Fig. 32 were somewhat less vigorous;

in the right scrotum there was an irregular indurated nodule with a depressed ulcer, and the flattened plaque on the left showed the merest shell of induration. Fig. 33 again shows a nodule or a lenticular thickening in the skin of the right scrotum which fades into the surrounding tissue. On the left, however, there is an extremely small but perfectly characteristic chancre. These photographs represent what was practically the highest point reached in the development of these lesions.

The next three illustrations of this group represent lesions which are decidedly less characteristic. The small ulcer in the right scrotum of the animal shown in Fig. 34 has a definite collar of induration, but on the left, there is little more than a minute sharply defined ulcer with a suggestion of a diffuse thickening in the scrotum. The next lesions (Fig. 35) consisted of irregular areas of thickening with even more irregular areas of ulceration. These lesions represented the height of the local reaction in this animal, and it may be of interest to note that generalized lesions appeared elsewhere just at this time (58 days after inoculation). The final photograph of the series (Fig. 36) shows small nodular lesions in the scrotum of both testicles which persisted for months with almost no change from the condition here shown.

Atypical and Diffuse Lesions of the Scrotum.

The lesions classed as atypical and diffuse include conditions varying from chronic ulcers and nodular thickenings in the scrotum to various inflammatory processes of an ill defined character. As a class, these lesions persist for a long time, and while there is usually no considerable difficulty in demonstrating the specific nature of the lesions, they are subject to frequent transformations which make it very difficult to say whether they should be regarded as primary or as secondary manifestations of infection, or where the line of separation between the two should be placed. At all events, the lesions are the same in either case, and since one group cannot be clearly differentiated from the other, they must be considered here without reference to their primary or secondary character.

Several groups of lesions belonging to this class have already been described and illustrated in Part 1 of this paper. These descriptions of atypical and diffuse scrotal lesions may be supplemented by further examples of lesions of a somewhat different type.

The photographs reproduced in Figs. 37 and 38 represent forms of primary scrotal lesions which were very commonly seen and frequently were the starting points for lesions such as those in Figs. 39 to 42. The first of these photographs

(Fig. 37) was taken 43 days after inoculation and shows a small indurated nodule with an apical ulcer in the right scrotum and an area of diffuse infiltration in the left. The skin in this area showed a loss of papillæ and increased translucency together with the formation of yellowish white scales over its central portion. The second photograph (Fig. 38) shows changes of an analogous character but somewhat more pronounced. The infiltration of the scrotum was more marked on both sides, and on the right there were definite areas of superficial necrosis and exfoliation. This photograph was taken 77 days after inoculation, and just at this time, patches closely resembling those in the right scrotum made their appearance upon the skin at the base of the ears. While neither set of the scrotal lesions shown (Figs. 37 and 38) might conform to the usual conception of a chancre, they were nevertheless the primary lesions of these animals and are just as characteristic of *pallidum* infection in the rabbit as any of the lesions previously described.

Fig. 39 represents another form of scrotal syphilide which occurred either as the starting point of an infection (primary lesion) or as a transformation of other types of lesions. This animal was kept under observation for 29 months after inoculation and never developed lesions more analogous to the ordinary chancre than those shown in Fig. 39, the photograph of which was taken 136 days after inoculation. Altogether, these lesions persisted in essentially the form represented for about 18 months and this animal showed a most marked generalized infection.

The lesions shown in Fig. 40 (190 days after inoculation) are a somewhat different form of the same process as that in Fig. 39. In this animal, there was a diffuse thickening of the scrotum of both testicles, most marked in the dependent portions. On both sides, there was a curved ridge (shown in the photograph only on the right) extending downward and spreading out into a flattened head at the lower end of the scrotum. This ridge and portions of its terminal expansion were profusely covered with scales and thin crusts with erosions here and there.

Finally, there are two illustrations (Figs. 41 and 42) of lesions which in their later transformations tended to revert to a form more like an ordinary chancre. The initial lesions of the animal shown in Fig. 41 were circumscribed nodular lesions with marked congestion and edema of the scrotum. These lesions were of short duration, and by the end of the 5th week after inoculation, they had almost disappeared, leaving a diffuse thickening of the scrotum analogous to that shown in Fig. 37 or 38. 94 days after inoculation (Fig. 41) there was involvement of both testicles (orchitis), diffuse thickening of the scrotum with the formation of fine bran-like scales (shown fairly well on the left), and indolent ulcers on both sides with thickening but no induration about them. These lesions persisted with some further transformations of an equally atypical character up to the time the animal was discarded 11 months after inoculation.

The last photograph of the series (Fig. 42) represents the lesions present in the scrotum of a rabbit 316 days after inoculation. The infection in this animal

began with the formation of lesions almost identical with those shown in Fig. 35 and remained a diffuse infiltrative and exfoliative type of process for upwards of 9 months. It was not until during the 10th month after inoculation that circumscribed lesions of the character shown in Fig. 42 began to appear. On the right, the lesions became confined almost entirely to the nodular mass at the lower end of the scrotum, but at least two-thirds of the left scrotum was the seat of a diffuse infiltration and at times showed a tendency to the formation of scattered exfoliative lesions. (Note the dark colored spots on the scrotum which represent areas of recent exfoliation.) In the midst of this diffuse process, the chancre-like lesion shown in the photograph was formed.

From the standpoint of the human infection, this group of atypical and diffuse lesions of the scrotum is of the utmost importance. They are in many instances lesions which at first glance do not suggest syphilis or at least primary syphilis. They have doubtless been noted by many observers, but very little attention has been paid to them, possibly for the reason that they were interpreted as evidences of a low grade or slight infection which is not necessarily the case.

We have had many rabbits with lesions of this type, a large proportion of which came from the Zinsser-Hopkins strain of *Treponema pallidum*. These animals were of no particular use for therapeutic experiments, and consequently only a comparatively small number of them was kept under observation for any considerable period of time. Those kept were held partly with the hope that they might eventually develop lesions which could be used and partly for the purpose of studying this particular class of infections. It was in this way that we learned what we have about them.

As a class, these atypical and diffuse lesions persisted fully as long or longer than any other class of primary lesions; they contained actively motile spirochetes in abundance, and these organisms were highly virulent which has been demonstrated in two ways, first by the fact that rabbits in which these lesions occurred were frequently the subjects of severe generalized infections, and next that organisms taken from such lesions also produced high grade infections when inoculated into other animals.

General Course of the Local Infection.

Judged upon the basis of the changes which took place in the lesions or by the reaction in the scrotum, the course of the scrotal infection appeared to be fundamentally the same as that in the testicle, modified undoubtedly by differences in the character of the tissue within which or from which the lesions developed. In the scrotum, as in the testicle, there were evidences of periodic changes, but the specific reaction in the scrotum was more stable than that in the testicle and less subject to marked or rapid changes in one direction or another. While one could distinguish between lesions or processes which were diffuse and those which were circumscribed, it was more difficult to draw a sharp line of demarcation between processes which were acute or exudative and those which were proliferative in character.

In general, the initial reaction in the scrotum usually progressed without interruption until a well defined lesion had been produced or for some 2 or 3 weeks at the least. The reaction then assumed an irregular character with periods of growth interrupted by longer or shorter intervals during which little or no change could be detected in the lesions, or during which the lesions appeared to regress. As long as the lesion was merely quiescent or inactive, it retained its appearance unaltered, but when regression set in, the skin about the lesion became relaxed and wrinkled, the induration softened, or the lesion diminished in size. At times, the entire lesion was affected by these changes, while at others, only certain parts of the lesion were affected, or, as in the case of the testicular infection, one portion of the lesion might be actively developing while another was rapidly regressing.

The time element in these changes was most uncertain—some lesions lay dormant over long periods of time (several months) and then grew actively, while others grew steadily for a long time before showing any sign of cessation of activity. Again one group of lesions would develop by more or less regular periods of growth interrupted by short intervals of inaction, while another would show the greatest degree of irregularity. As a rule, a period of growth lasted for a week or more before it was interrupted by an interval of inactivity.

The extent of the change which took place during one of these periods of growth or regression was also a matter of great variation. Growth might be rapid or extremely slow, so rapid that a chancre measuring 2 or more cm. in diameter would develop within as many weeks from the commencement of the reaction, or so slow that no change could be detected from week to week and growth was recognizable only by the change which took place from month to month. Conversely, the phase of regression might be limited to inaction only or might go so far as almost to obliterate the lesion which had been developed and still be followed by an active renewal of growth. However, marked changes in one direction followed by marked changes in the other were extremely uncommon except in cases in which the complete life cycle of the lesion consisted of one such series of changes or was the terminal change in the local infection.

While the extent of the cyclic change was usually limited to a moderate reaction in one direction or the other, the number of such cycles was at times very great, especially in lesions which developed slowly and persisted over long periods of time. As a rule, however, the complete series of such cycles did not exceed three or four and not infrequently was limited to a single cycle.

The duration of the infection in the scrotum as a local or primary focus of infection may be variously stated as from 1 to 18 months. In a limited number of cases the infection developed quickly and subsided with equal rapidity or there was but slight local reaction and this soon subsided. At the other extreme, there were infections which remained firmly established for more than a year, but the average duration of the scrotal chancre was hardly more than 4 to 6 months.

In the majority of instances, the infection terminated by degrees. Development gradually ceased and after remaining in a more or less stationary condition for a time, the lesion gradually underwent resolution or healing with the production of a scar, the process consuming on an average from 4 to 6 weeks.

Spirochete Content of Scrotal Chancres.

To demonstrate the presence of spirochetes by the aspiration of fluid from scrotal lesions is usually a very simple operation, but owing to differences in the character and structure of the lesions, comparisons of the numbers of spirochetes in different examples or in different portions of the same lesion are always open to some question. Thus spirochetes are obtained with comparative ease from lesions of a cellular or mucoid character but are more difficult to obtain from fibrous lesions. Again, calculations may be considerably upset by the simple element of dilution which becomes of especial importance where edema exists, and in any case, a negative examination has only a relative significance.

It seems well to emphasize the importance of these points, since in therapeutic experiments, so much stress has been laid upon the number of spirochetes present in lesions at the time of treatment and upon the relative reduction in the number of spirochetes produced by different therapeutic agents. From a wide experience, we realize that no small part of such differences may be traceable to just such factors as those which have been enumerated. This is especially applicable to effects attributed to drugs which produce a marked increase in the fluid content of lesions as a characteristic feature of their action, and, we may add, there are many such drugs, and these are the ones which, as a rule, produce the most striking apparent reductions in the number of spirochetes present.

With these facts in mind, it may be said that during the early period of chancre growth and as long as a uniform and continuous growth was maintained, actively motile spirochetes could be found in abundance throughout the lesion. Eventually the spirochete content became variable as in other primary lesions. They were numerous and actively motile during periods of active growth or even quiescence, but decreased or even disappeared temporarily from the aspirated fluid during periods of actual regression.

Spirochetes varied likewise with developmental processes or with pathological alterations taking place in different portions of the lesion. As central necrosis developed, they became less numerous towards the center of the lesion or less active and tended to accumu-

late in greatest numbers towards the outer or growing edge. In well developed lesions with necrotic but firm fibrous centers, spirochetes were obtained with difficulty from the central zone and might be obtained only from the outer shell of living tissue. However, in chancres which underwent central softening following necrosis, spirochetes were at times quite numerous in the necrotic debris.

With the progress of the infection and the appearance of irregularly distributed areas of growth and of necrosis, the distribution of spirochetes became likewise irregular; they tended to be numerous and actively motile in regions of most active growth, less numerous and less active in portions of the lesion which were inactive or regressing.

The relation of the spirochete to the size and character of the lesion was less definite. Spirochetes were as abundant and as actively motile in some of the smallest and most insignificant lesions as in the largest and most typical lesion. As a rule, spirochetes were obtained in greater numbers by aspiration of cellular and mucoid lesions or portions of lesions than from fibrous lesions or areas, but, as previously stated, this does not necessarily represent actual differences in the spirochetel content of the respective lesions.

Results Obtained from Scrotal Inoculation.

Before concluding the subject of the scrotal infection in the rabbit, it seems well to refer briefly to the results which one may obtain from scrotal inoculations with *Treponema pallidum* and certain factors which influence these results.

As has already been indicated, there is no particular difficulty in obtaining an infection with organisms which have been thoroughly adapted to the rabbit. With such organisms as we have used, 100 per cent of takes can be obtained with perfect regularity provided one observes a few simple precautions which concern chiefly the state of the virus, the animals used, and the technique of inoculation. As regards the character of the local infection, or more properly the local reaction, uniformity is more difficult to attain, but this also is influenced to a considerable extent by the same factors.

It has been found that the first essential to success is the use of a suitable virus. As far as mere infection is concerned, material taken from any lesion containing a fair number of actively motile spirochetes will produce infection, but the character of the infection will vary according to the state of the lesion or of the spirochetes in the lesion. Other conditions being equal, the local reaction will take place more promptly and large actively growing chancres will be produced with greater regularity when the material for inoculation is taken from a fresh actively growing lesion, preferably before the height of the first cycle of reaction has been reached. Material taken from lesions during the ascending phase of the second cycle of an orchitis or of later cycles in the case of skin lesions will sometimes give good results, but at other times, the results are apt to be irregular and less satisfactory as far as the production of chancres is concerned.¹

In addition, it should be noted that certain strains of *Treponema pallidum* will give results which are more constant and more satisfactory than the results obtained with other organisms.

The second factor in the production of scrotal chancres is the character of the animal used. The proverbial large rabbit or rabbit with large testicles which is usually interpreted as an old rabbit with a more or less ample or redundant scrotum is a poor subject for the production of scrotal chancres. The best animals are those just approaching full maturity with well developed but not necessarily large testicles and a thin delicate scrotum. It is also desirable that the rabbits used be well nourished and free from disease.

Among the various breeds of rabbits, there are great differences in the scrotal reaction to *pallidum* infections. The Belgian and Flemish giants in particular give poor results, while the small albinos, grays, browns, and Dutch belts on the whole give results of the most satisfactory character.

¹ It is not to be inferred that the extent of the local reaction in the scrotum is an index of the severity of the infection as a whole, for, as has been pointed out, some of the most severe generalized infections follow the most insignificant local reactions. Further, there is evidence to the effect that in general a pronounced local reaction in the testicles or scrotum of the rabbit inhibits the development of other focal infections. This subject will be dealt with in detail in a subsequent paper.

Technique is another factor which plays some part in the success of scrotal inoculations, and the chief element here is simplicity. No antiseptics is necessary, and the use of strong antiseptics is distinctly contraindicated both on account of irritant action upon the scrotum and the possible effect of these substances upon the spirochetes themselves. Many failures in scrotal inoculations have been directly attributable to unnecessary precautions in this direction. Cleanliness, careful operation, and avoidance of undue trauma are the three essentials.

With the use of a suitable virus, good animals, and proper technique, one may attain a high degree of uniformity from scrotal inoculations in as far as the percentage of takes and the production of typical scrotal chancres is concerned. Large indurated chancres were frequently obtained by us in from 75 to 100 per cent of the animals inoculated, but as a rule such lesions were produced in not more than 50 to 75 per cent of the animals. The factor of individuality in the reaction to infection cannot be entirely overcome in *pallidum* inoculations. Just as the typical chancres in a given series of animals will differ from one another, so also one may expect to obtain all degrees of variations in the response of individual animals to a constant set of conditions of inoculation, and irregularities will appear in the results in spite of all that can be done to prevent them.

CONCLUSIONS.

A study of the local infections produced by *Treponema pallidum* in the testicles and in the scrotum of the rabbit leads to the conclusion that the phenomena of infection in the two cases are essentially the same and that such differences as do exist are attributable to differences in the character of the two organs. Upon the basis of these studies, it is possible, therefore, to extend the conclusions which have already been reached in regard to the nature of the local or primary reaction to infection with *Treponema pallidum*.

One is accustomed to think of this reaction as essentially a process of infiltration and proliferation and of the chancre as a circumscribed, indurated, granulomatous lesion. From a consideration of the facts derived from a study of the local infection in both the testicles and the

scrotum, it is apparent, however, that in the rabbit at least, this conception of the syphilitic infection is true only in part and that it is derived more from a study of a condition accomplished than from the process concerned in the production of this condition.

It would seem that there are concerned in the local reaction to infection an element of toxic injury with subsequent degeneration and necrosis which affects especially the lymph and blood vascular systems, a process of exudation and infiltration, and finally proliferation and a mass necrosis due to a progressive destruction of the vessels supplying the affected area. While infiltration, proliferation, and necrosis are the most noticeable features of the local reaction in the usual case of infection, they are, however, no more characteristic than the other processes mentioned and appear to be phenomena of secondary character and importance.

None of these changes bears a fixed relation to the infection, but they are subject to the widest possible variations and in consequence give rise to lesions of the most diverse character in all of which evidences of the same fundamental processes are to be seen. As one feature or another of the reaction becomes more marked, the character of the lesion changes accordingly. Thus the lesion produced in different animals or in the same animal at different periods of the infection may range from lesions in which congestion, edema, and even hemorrhage are the most prominent characteristics to massive granulomatous lesions on the one hand, and from diffuse or ill defined patches of infiltration with desquamation or exfoliation of the surface epithelium to the most sharply circumscribed and intensely indurated nodules on the other.

Finally, it may be said that there is nothing so far as we have been able to discover which clearly differentiates the local reaction at the primary focus of infection from localized reactions to a generalized infection, unless it is the one element of the intensity of the reaction. In many instances, even this distinction is lost, and, as will be brought out in subsequent papers, one reaction is but a repetition of the other, modified to a greater or less extent by the general reaction opposed to the infection and the character of the tissues within which the reaction takes place.

SUMMARY.

From a study of the reaction to scrotal inoculation with *Treponema pallidum* in a large series of rabbits, it was found that the specific reaction presented the following characteristics.

In general, the reaction in the scrotum became apparent within 7 to 14 days after inoculation but was subject to considerable variation. The early reaction took the form of an edematous swelling and congestion associated with a new growth of vessels or of an infiltration with more or less proliferation of fixed tissue cells. These reactions were either confined to a small circumscribed area of the scrotum or were of a diffusely spreading character, and as the infection advanced, the infiltration and proliferation together with such secondary changes as exfoliation, necrosis, and ulceration became the most conspicuous features of the reaction.

The course of the reaction in the scrotum was essentially the same as that in the testicle; that is, it was periodic in character and was marked by a phase of active progression followed by quiescence or regression and renewed activity.

The scrotal reaction resembled that in the testicle also in the varying character of the reaction, appearing at times as a circumscribed focus of reaction and later becoming diffuse, or first as a diffuse reaction which subsequently became more localized.

The lesions produced in consequence of this reaction were of two general types—one a circumscribed indurated granulomatous lesion closely resembling the human chancre, the other a diffuse infiltration more analogous to the secondary skin lesions of man. Both groups of lesions presented the greatest degree of individual variations and possessed no fixed status but were subject to frequent and marked transformations. After a period of from a few weeks to many months, the lesions in the scrotum disappeared spontaneously.

EXPLANATION OF PLATES.

All the illustrations are reproductions of untouched photographs which represent the objects at their natural size. The statements of time given are estimated from the date of inoculation except where otherwise stated.

PLATE 83.

FIGS. 1 to 6. Typical nodular chancres of the scrotum.

FIG. 1. 49 days. Characteristic multinodular chancres with small depressed ulcers.

FIG. 2. 41 days. Intensely indurated chancres with marked central necrosis. Edema and congestion of the scrotum with focal hemorrhages on the left.

FIG. 3. 44 days. Nodular chancres with unusually marked central necrosis and only a comparatively thin shell of living tissue. These features were developmental characteristics.

FIG. 4. 44 days. Large nodular chancres with unilateral flattening. Growing surfaces slightly mucoid in character. Edema of the scrotum.

FIG. 5. 44 days. Large nodular chancres with marked surface flattening of the right chancre due to spreading necrosis.

FIG. 6. 70 days. A vigorous nodular chancre on the left with marked central necrosis of the chancre on the right—a phenomenon of decadence.

PLATE 84.

FIGS. 7 to 12. Nodular chancres of a more spherical type with a lessened tendency to necrosis and ulceration.

FIG. 7. 43 days. Spherical chancres. Spreading surface necrosis on the right, slight necrosis on the left.

FIG. 8. 46 days. Spherical chancres ulcerated and un ulcerated.

FIG. 9. 60 days. Spherical chancres. The skin surface on the left still uninvolved.

FIG. 10. 90 days. Irregular nodular chancres with small areas of skin necrosis.

FIG. 11. 89 days. Spherical chancres in the subcutaneous tissues of the scrotum, with no sign of surface necrosis. Diffuse infiltration of the lower end of the scrotum on the left.

FIG. 12. 112 days. Spherical chancres in the deeper layers of the skin with infiltration of the papillary layers but no necrosis.

PLATE 85.

FIGS. 13 to 18. Typical lenticular and discoid chancres of the scrotum.

FIG. 13. 28 days. Rapidly growing lenticular chancres with spreading necrosis of the skin surface. Thick acuminate crusts.

FIG. 14. 28 days. Discoid chancres with deep necrosis and ulceration on the right.

FIG. 15. 38 days. Discoid chancres with deep ulceration and thick, dry crusts. Marked edema of the scrotum.

FIG. 16. 45 days. Typical mucoid chancre with extensive ulceration and infiltration of the skin.

FIG. 17. 39 days. Discoid chancre of more fibrous type.

FIG. 18. 46 days. Discoid chancres with spreading necrosis and thick imbricated crusts.

PLATE 86.

FIGS. 19 to 21. Growth and necrosis of mucoid chancre.

FIG. 19. 45 days.

FIG. 20. 65 days.

FIG. 21. 86 days.

FIGS. 22 to 24. Evolution of a more spherical mucoid chancre.

FIG. 22. 39 days.

FIG. 23. 46 days. Necrosis beginning to spread.

FIG. 24. 60 days. Spreading necrosis with thick imbricated crusts.

PLATE 87.

FIGS. 25 to 30. Modified types of lenticular and discoid chancres.

FIG. 25. 35 days. Lenticular mucoid chancres.

FIG. 26. 40 days. Mucoid chancres tending towards a spherical form.

FIG. 27. 61 days. Lenticular chancres of dense fibrous structure.

FIG. 28. 65 days. Lenticular chancres of dense fibrous structure showing marked necrosis and thick adherent crusts.

FIG. 29. 37 days. Mucoid chancres with moderate induration, slight skin involvement, and undermining necrosis (right).

FIG. 30. 97 days. Old fibrous chancres with undermining necrosis and relaxed skin covering. Inactive.

PLATE 88.

FIGS. 31 to 36. Transitional types of chancre reactions.

FIG. 31. 84 days. Right, a flattened plaque of induration with ulceration extending practically to its edges. Left, an irregular area of induration, necrosis, and ulceration. Both chancres still quite active.

FIG. 32. 73 days. An irregular ulcerated nodule on the right and a small flattened chancre on the left.

FIG. 33. 55 days. Irregular mass of induration on the right and a very small but typical lenticular chancre on the left.

FIG. 34. 36 days. Small lenticular chancre on the right and a small patch of necrosis in the skin on the left.

FIG. 35. 58 days. Irregular patches of necrosis and ulceration with slight infiltration of the surrounding tissues.

FIG. 36. 29 days. Small nodules of induration.

PLATE 89.

FIGS. 37 to 42. Atypical scrotal lesions (chancres (?)).

FIG. 37. 43 days. Right, a small indurated papule with central ulcer. Left, an area of diffuse infiltration and slight desquamation of epithelium.

FIG. 38. 77 days. Diffuse infiltration with superficial necrosis and exfoliation.

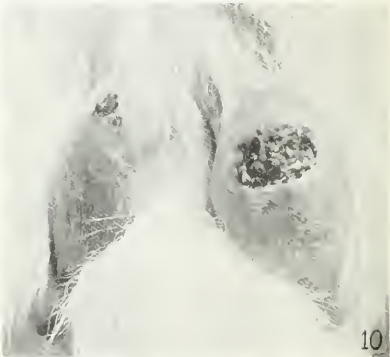
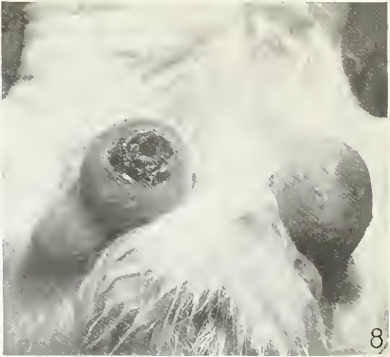
FIG. 39. 136 days. Marked infiltration of certain areas of the scrotum with focalized areas of necrosis and ulceration and extensive exfoliation.

FIG. 40. 190 days. Diffuse infiltration of the scrotum with marked exfoliation in certain areas.

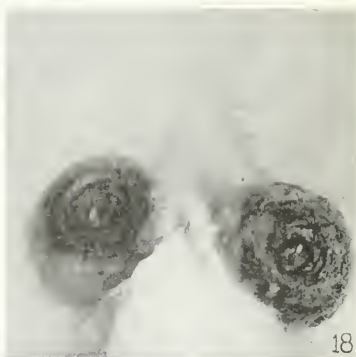
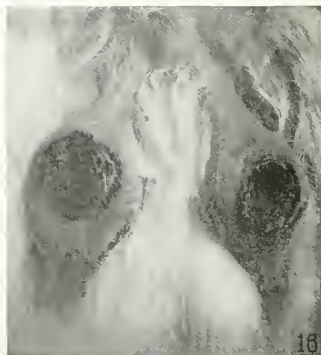
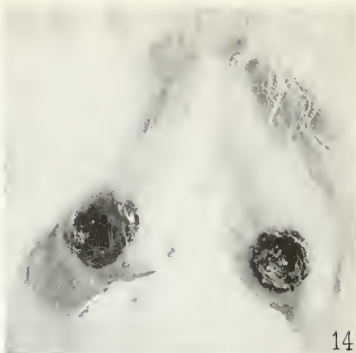
FIG. 41. 94 days. Diffuse infiltration of the scrotum, secondary ulceration with slight induration, and double orchitis.

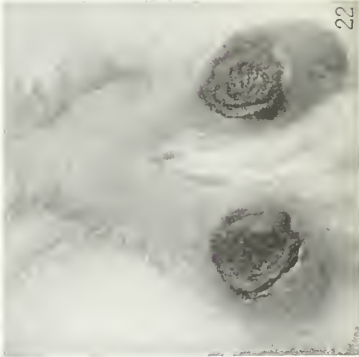
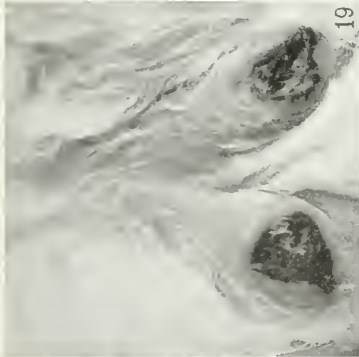
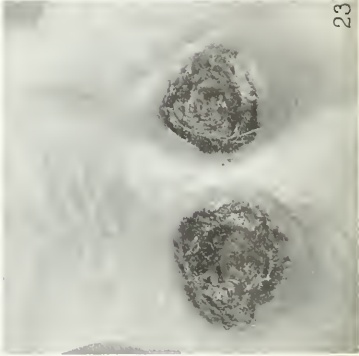
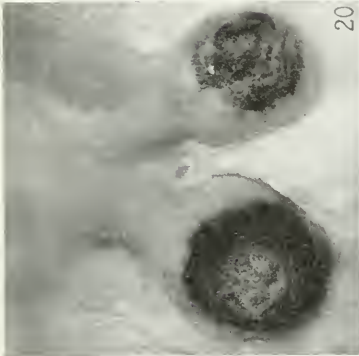
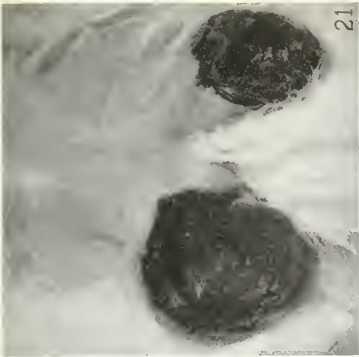
FIG. 42. 316 days. A late reversion of a diffuse scrotal syphilis to lesions of a circumscribed type.

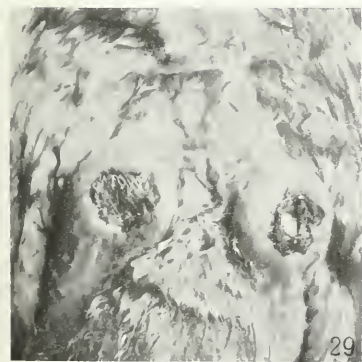


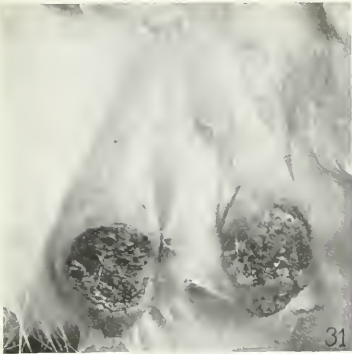


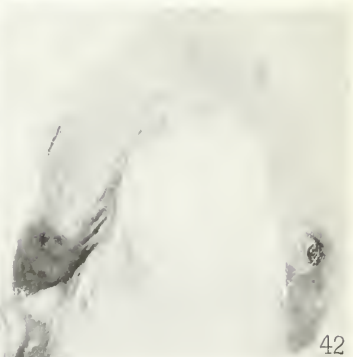
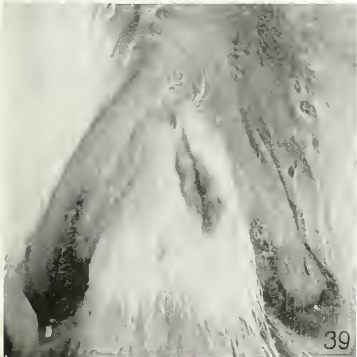
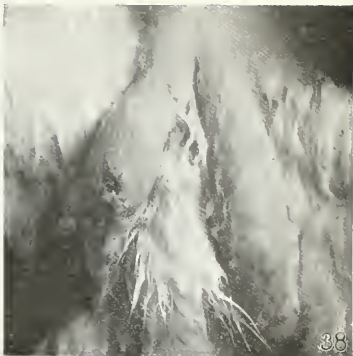
(Brown and Pearce: Experimental syphilis in the rabbit. 11.)











EXPERIMENTAL SYPHILIS IN THE RABBIT.

III. LOCAL DISSEMINATION, LOCAL RECURRENCE, AND INVOLVEMENT OF REGIONAL LYMPHATICS.

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PLATES 90 TO 97.

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In preceding papers of this series, the testicular and scrotal infections of *Treponema pallidum* were described from the view-point of a progressive local infection, and in following out this mode of presentation, a number of lesions were described as transformations or extensions of the local infection without any attempt to differentiate such lesions from lesions of a similar character which might arise as a result of local dissemination of the infection rather than from direct extension or transformation of primary lesions. It was pointed out, however, that there were many lesions of the testicles and scrotum which occupied a position of uncertainty with reference to the primary infection and that it could not be determined in all cases whether the lesions present represented actual transformations and extensions of a primary infection or had arisen as a result of local dissemination.

In brief, it was recognized that there were two groups of lesions in the testicles and scrotum which could not be clearly separated from one another. One group is composed of modified and persistent forms of primary lesions together with direct extensions and true recurrences, while the other represents lesions which owe their origin to local or regional dissemination of the virus.

These lesions are of especial interest in that they mark a transition between conditions which can be clearly recognized as primary manifestations of infection and conditions which represent true generalized infection. The subjects of chief interest in this connection are those of local spread and local dissemination of the infection, recurrent and

consecutive lesions, and involvement of regional lymphatics. References to this phase of experimental syphilis have been very few and for the most part rather vague.

Attention was called to the phenomenon of local metastasis by Uhlenhuth and Mulzer and by Truffi at about the same time. Truffi (1) reported a case of a granulomatous nodule arising in the epididymis and another of a lesion in the tunics following primary infections of the scrotum. Uhlenhuth and Mulzer (2) reported metastatic infection from one testicle to the other testicle and scrotum and a second case of metastasis to an uninoculated testicle after removal of the inoculated testicle. These observations were made in 1909 and 1910, and since that time, metastases of this kind have frequently been cited as evidence of generalization of the infection in the experimental animal.

Involvement of the inguinal lymph nodes in infected animals has likewise been noted both as an analogy to the human infection and as proof of generalization. This condition was first reported by Ossola (3) (1909) who was able to demonstrate spirochetes in sections of the inguinal nodes of animals inoculated in the scrotum. These findings were confirmed by Truffi (4) and the virulence of the organisms was proven by animal inoculation.

Other than observations of this kind, very little has been recorded concerning this border-line group of syphilitic manifestations.

Local or Regional Dissemination.

In animals infected with *Treponema pallidum*, one must recognize the existence of two opposing influences, the tendency of the organism to spread beyond the local confines of the point of inoculation and the opposing reaction on the part of the animal. Some extension of the infection and of the zone of local reaction occurs in all animals even with the most sharply circumscribed lesions, but the extent of the two processes does not run parallel.

The details of the local changes are either masked in a continuous and widespread reaction or else there are no lesions to mark the extension of the infection beyond the zone of primary reaction. In exceptional instances, however, cases do arise which when linked together furnish some interesting data concerning the spread, or dissemination, of the infection within the regions adjacent to the point of inoculation.

In addition to the widespread reaction commonly observed in the course of development of primary lesions, there were three general

types of local reaction which we were able to make out in connection with the dissemination of the infection in the testicles and scrotum: first, a diffuse reaction with the formation of multiple foci of secondary reaction arising subsequent to the development of the primary lesion; second, a reaction characterized by the formation of lesions having a perivascular arrangement; and third, dissemination of the infection with the production of multiple lesions showing no demonstrable connection with the primary focus of infection. While lesions of these three classes occurred in both the scrotum and testicles, they were most clearly definable in the scrotum, and for that reason, the discussion of the subject will be confined to the scrotal infection.

Diffuse Secondary Reaction with the Formation of Multiple Focal Lesions.—One of the most common forms of local dissemination observed was one which took place with a more or less diffuse reaction about a primary focus of infection and led to the formation of multiple secondary lesions which tended to become fused into a single chancre-like mass. This form of reaction is apt to be confusing and it is only in cases where the successive steps in the process can be followed that the ultimate lesion can be distinguished in any way from a true primary lesion. A typical case of this kind is illustrated in Figs. 1 to 3.

The primary lesions in this animal were small circumscribed chancres of a rather indolent character and for approximately 2 months there was nothing unusual about the local infection. A slight diffuse thickening of the scrotum then developed about these lesions and a number of small thickened plaques were formed here and there in the scrotum. These plaques developed into nodules and the diffuse reaction in the scrotum increased after the manner shown in Fig. 1. At the time this photograph was taken, 85 days after inoculation, the primary lesions themselves (indicated by arrows) had begun to grow actively; the entire scrotum was markedly thickened and slightly reddened, and the focal lesions showed signs of necrosis and ulceration. The development of these lesions continued as indicated by Figs. 2 (91 days after inoculation) and 3 (113 days after inoculation) with the formation of an increasing number of secondary foci which tended to fuse into a single large granulomatous lesion indistinguishable in character from an ordinary chancre.

It should be noted here that the development of this series of lesions in the scrotum, occurring 2 months after inoculation, coincided with the appearance of similar granulomatous lesions on the

feet and legs of this animal and the two sets of lesions ran a parallel course of development.

This is but one of many cases of the kind which have come under our observation but will serve to indicate the possibilities for confusion of primary lesions and lesions which owe their origin to dissemination from a primary focus of infection.

Local Dissemination with the Formation of Perivascular Lesions.—The second form of local dissemination to be considered differed from the first chiefly in that the development of the lesions was less obscured and the lesions themselves were of a very distinctive type.

In taking up this group of lesions, we may refer first to a form of direct extension from the primary lesions which was observed in cases in which the initial lesion was slow in developing or of slight extent and spread of the infection along the perivascular tissues could be detected even before the reaction at the site of inoculation had become well established. The animal shown in Fig. 4 was one of a group inoculated with a virus known to contain large numbers of spirochetes of low vitality. Following inoculation, the implant became soft and cheesy in character and no specific reaction could be detected for about 6 weeks. Finally, a narrow line of induration developed at the outer margin of the implant in the right scrotum and fine thread-like lines of infiltration immediately began to spread along the course of the blood vessels leading away from this area as shown in the photograph (Fig. 4) taken 56 days after inoculation. These lines of infiltration grew until they formed a series of cords about 1 mm. in diameter with node-like thickenings at various points. Later, the intervening sections in the distal portions of the cords disappeared, leaving a series of isolated shotty nodules, while the parts nearest the implant became fused into a single irregular granulomatous mass.

In the left scrotum, a similar type of reaction took place except that the lines of perivascular extension were not so clearly defined. When first discovered, there were numerous isolated points of induration diffusely scattered through the scrotum, many of them occupying a perivascular position. Soon after the appearance of these disseminated lesions in the scrotum, lesions developed upon the sheath and about the anus of this animal.

We have in this animal what appears to be a twofold process, first a gradual but direct extension of the primary reaction along certain definite lines, and second a widespread development of focal lesions resulting from dissemination of spirochetes with which the development of the primary lesion had not kept pace.

A somewhat clearer example of local dissemination with the production of perivascular lesions is to be found in the animal shown in Figs. 5 and 6 which belonged to the same series as that shown in Figs. 1 to 3. The initial lesions were again small ulcerated chancres with slight induration. These lesions were soon transformed into a moderate diffuse thickening of the ventral surface of the scrotum, while the ulcers healed, leaving small puckered scars (Fig. 5). About 6 weeks after inoculation, a series of small shotty nodules was palpable in the dorsal fold of the scrotum. These nodules were just visible as translucent points distributed in chains along the course of the blood vessels extending centrally from the thickened patches in the scrotum. They gradually increased, and at the time the first photograph was taken (Fig. 5, 63 days after inoculation), there were numerous nodules distributed all through the scrotum, including the area beneath the scar, and the course of the blood vessels in the dorsal fold was marked by a series of indurated lines and nodes (Fig. 5). While these lesions are shown on only one side, the condition on the other was exactly the same.

During the next 3 weeks, the character of the lesions changed materially. Many of the papular lesions disappeared, leaving only a few perivascular lesions in the dorsal portion of the scrotum (Fig. 6). The chief seat of the specific reaction shifted back to the ventral surface of the scrotum which showed a heavy shell of doughy thickening with a few large masses of induration. The subsequent changes were much the same as those shown in the first series of photographs (Figs. 1 to 3).

Again we may note that the occurrence of these papular lesions in the scrotum coincided with the development of a periosteal lesion at the distal end of the left ulna.

While lesions of the type described were observed in all parts of the scrotum, the seat of predilection was the lower end of the dorsal fold, and the occurrence of one or more lesions in this location was quite common. When the lesions were multiple or in the form of cords, they frequently followed a definite line of distribution or extension from the point of inoculation or from the primary lesion towards the body axis.

Local Dissemination with the Formation of Multiple Secondary Lesions.—The third group of disseminated lesions to be described differed from the first group only in that they were clearly focalized lesions from the beginning and from those of the second group in that they showed no apparent perivascular relation and no definite connection with the parent lesion. These lesions usually appeared as multiple papules or plaques and tended to develop into multiple lesions of a chancre-like character. Sometimes there were only one

or two such lesions, but at others, there were as many as six or eight. The animal shown in Fig. 7 had six distinct lesions of different sizes and stages of development in each scrotum, among which it would be rather difficult to identify the primary lesion. As in the previous cases, they tended ultimately to diminish in number, and the reaction usually became centered in one or two lesions which developed to a considerable size, overgrowing and fusing with the surrounding lesions.

It is probable that lesions of this class have in general been regarded as multiple primary lesions, but as a rule they do not begin to appear until after a primary lesion of some sort has developed at the immediate point of inoculation or for some 6 weeks or more after inoculation, and while they may become the main lesions present, they are not primary in the sense of lesions developing as a result of simultaneous localization of the infection. This distinction may or may not be of importance according to the significance which may be attached to primary and so called secondary lesions.

Recurrent Lesions.

The lesions which develop in the testicles and scrotum of an infected animal subsequent to the regression or healing of an initial lesion have many points of interest, but their chief importance lies in their bearing upon problems of local dissemination and the persistence of active infection when once the local lesions have completely disappeared. In its application to experimental therapy, there are few subjects of greater importance than this, and while we cannot enter into this phase of the subject with the thoroughness which it demands, the therapeutic importance of recurrent lesions may be kept in mind.

The lesions which develop after spontaneous healing of an initial lesion and those which appear after healing which has been induced by artificial means are identical in all respects. Induced healing, as by the use of therapeutic agents, offers the advantage, however, of clearer definition between the process of healing and recurrence, and for this reason we shall use cases for illustration in which the healing of the original lesion was experimentally induced.

As an introduction to the subject of true recurrence, it may be pointed out that the power of recovery in an early and actively developing scrotal chancre is very great and that regression may be carried almost to the point of complete healing and be followed by prompt regeneration of the lesion. This fact is illustrated in Figs. 8 to 10. Fig. 8 shows two chancres 40 days after inoculation at which time the animal was treated. The lesions regressed rapidly, and 2 weeks later, there was an irregular thickened mass in the right scrotum and only a small ulcer with no induration in the left (Fig. 9). At this point, regeneration set in, and in 2 weeks more, chancres showing the same characteristics as the original lesions were produced (Fig. 10). This is the result usually obtained when regression stopped short of complete healing.

Once complete healing of a lesion was accomplished, it was rare that another lesion developed from exactly the same point. The four most common locations for recurrent lesions were the tissues beneath the scar, the edges of the scar, the dorsal folds of the scrotum, and the testicles. The lesions developing in the scar were usually no more than indurated plaques, although typical chancres were observed which formed upon the basis of an old lesion.

In the tissues beneath the scar, the recurrent lesions usually took the form of indurated nodules, some of which might develop to considerable size and involve the overlying skin with the formation of chancre-like lesions.

A typical example of this form of recurrence is given in Figs. 11 to 13. Fig. 11 is the original chancre 29 days after inoculation, Fig. 12 the healed lesions, and Fig. 13 the multinodular recurrence with an ulcerated chancre-like lesion in the left scrotum 78 days after treatment.

A more common form of deep scrotal recurrence is that shown in Figs. 14 to 16. Recurrence in this animal did not take place until 55 days after treatment, and the lesions formed were small indurated nodules which were at first confined to the subcutaneous tissues. Later, the skin became involved, and a diffuse induration with multiple focal lesions extended over a large portion of the scrotum of both testicles (Fig. 16). During 4 months observation, no typical chancre-like lesion developed.

Another form of recurrence which was frequently seen in the scrotum is that shown in Figs. 17 to 19. This animal was treated 37 days after inoculation and the chancres healed promptly. 55 days after treatment, there were a few small indurated nodules at the outer edges of the scars on both sides. The scars themselves were smooth and thin (Fig. 19, 77 days after treatment). Subsequently other lesions of the same character appeared elsewhere in the scrotum, but they remained for only a short time and none of them developed to a size much larger than that shown in the photograph.

In some instances these recurrent lesions even though they were far removed from the seat of the original chancre showed a form of development identical with that of a chancre. Such a case is illustrated in Figs. 20 to 22. Recurrence took place in this animal 127 days after treatment, and the lesions appeared as single, minute, indurated nodules situated at the lower end of the dorsal fold of the scrotum; they were bilateral and symmetrical. These lesions grew rapidly and assumed the appearance of typical chancres (Fig. 22, 133 days after treatment).

In addition to the various forms of nodular lesions described, diffuse lesions of various types were observed in many animals. A case of this type which combines something of the characteristics of a diffuse and a focal process is shown in Figs. 23 to 25. This animal was treated 48 days after inoculation (Fig. 23). The lesions regressed very rapidly until they had almost healed, when a diffuse infiltration appeared over a large part of the scrotum (Fig. 24). Healing of the initial lesion continued, however, with the formation of an ordinary looking scar, and the thickening of the scrotum diminished (compare Figs. 24 and 25). 28 days after treatment, a series of slightly reddened and translucent points appeared around the margins of the scars (Fig. 25, 35 days after treatment). These spread rapidly, producing a slight diffuse induration in the scrotum with scaly points here and there.

A final form of recurrence to be noted is that which takes place in the testicle following the healing of a scrotal lesion, and it may be mentioned in this connection that such recurrences were quite common as were recurrences of testicular lesions in the scrotum. The most common locations of recurrent lesions in the testicle were the head of the epididymis, the tail of the epididymis, the mediastinum testis, and the tunics, or the same positions in which primary testicular infections tend to become localized.

This form of recurrence is illustrated in Figs. 26 to 28. The chancres in the scrotum of this animal healed completely in 3 weeks. 1 week later, there was a definite swelling of both testicles and indurated nodules were found in the head of the epididymis on both sides (Fig. 28); the scrotum remained normal.

The time of occurrence and the relative frequency of the various types of recurrent lesions will not be entered into. It seems well to mention, however, that the more characteristic lesions usually appeared early after the healing of the original lesion, while the smaller, less easily recognizable ones were more delayed in their development. The time of recurrence in our series of animals varied anywhere from a few days to many months, and so many circumstances enter into

estimations of the possibilities of local or regional recurrence that definite statements as to time limits cannot be made upon the basis of the data at present available.

To emphasize the difficulties attendant upon observations as to recurrence of local infections, the case illustrated in Figs. 29 to 31 may be cited. Following treatment, the chancres in this animal (Fig. 29) healed rather slowly, and healing was not complete at the end of 6 weeks (Fig. 30); however, smooth, thin scars were eventually formed. 63 days after treatment, two small areas of thickening appeared at the edge of the scar in the right scrotum but disappeared within a few days and infection could not be proven. 105 days after treatment, the extremely small nodule shown in Fig. 31 appeared in the left scrotum and proved to be syphilitic. There was a slight local edema associated with this nodule, but the nodule did not increase in size and persisted for only a short time. No other local lesions were found, but the animal developed a specific iritis 192 days after treatment.

A second animal of a similar character which came under our observation was one in which minute points of induration appeared in the dorsal folds of the scrotum 107 days after treatment. These nodules persisted for 8 months with very little change and no other lesions appeared for some time, but eventually a generalized rash developed upon the face and ears.

It will be noted that the lesions described as recurrences are not unlike some of the conditions previously described in connection with transformations of primary lesion and especially like those resulting from local dissemination. In fact, transformation, dissemination, and recurrence are all overlapping processes with no sharp line of separation between them, and each of these phenomena throws some light upon the others.

Involvement of Regional Lymphatics.

Enlargement and induration of the inguinal lymph nodes has been noted by several observers in connection with *pallidum* infections of both the testicles and the scrotum of the rabbit, and organisms have been demonstrated in these nodes in some instances. There is, however, little definite information concerning the relation of lymphadenitis to the experimental infection. The subject is a very broad one in itself and will not be discussed in detail at the present time. This aspect of the problem of experimental syphilis will be taken up in a separate paper, and we shall confine ourselves here to

general statements concerning involvement of the inguinal nodes in primary infections of the testicle and scrotum as a phase of local dissemination.

In normal rabbits, the inguinal glands are small, ovoid, or flattened masses which are barely visible or palpable (Fig. 32). From examination of a large number of rabbits, it was found that enlargement and induration of these nodes was inconstant in testicular infections and depended largely upon the extent of the local infection. In cases in which localized infection was confined to the testicles proper, there was little or no involvement of the inguinal glands, but when the infection extended to the scrotum, these glands became involved just as in cases of primary scrotal infection.

Character of the Involvement.—Localized infection in the scrotum was found to be invariably associated with marked inguinal lymphadenitis. Following inoculation or extension of an infection to the scrotum, pathological changes in the inguinal nodes could be detected almost immediately. The change began as an acute swelling, part of which might have been due to a non-syphilitic reaction but was merged indistinguishably with the syphilitic reaction which progressed rapidly until the affected glands were several times their normal size. During the early stages of the enlargement, the glands remained rather soft but gradually became harder and finally reached a condition of extreme induration. In some instances, the stage of acute swelling was absent; in these cases, the glands first became hard and shotty and then underwent a gradual enlargement.

The exact change which occurred in individual animals was found to be very variable, but a general idea of the inguinal adenopathy may be gained from an examination of the photographs reproduced in Figs. 32 to 43 which represent various forms and degrees of involvement.

As may be seen by reference to Figs. 33 and 34, a well developed adenitis was present in cases of active infection within a few days after inoculation. The 5 day example given in Fig. 33 is a rather pronounced instance of acute bilateral swelling more marked on the right than on the left. In Fig. 34 (7 days after inoculation), the change is not so apparent, especially on the right. Upon close examination, however, it will be seen that there are three nodes in this region, the largest of which is situated immediately over the cord and hence does not stand out so prominently as the one on the left.

The phase of acute swelling as exemplified in these two illustrations frequently reached its height by the end of the 1st or 2nd week after inoculation. The induration, however, continued to increase for some time and was not infrequently associated with a slight temporary decrease in the size of the gland. When fully developed, the inguinal adenopathy presented a variety of appearances. The glands chiefly affected were those immediately adjacent to the inguinal ring. As a rule, the involvement was well marked and of the same character on the two sides but rarely equal (see figures); occasionally the nodes of one side were much more enlarged and indurated than those of the other (Fig. 36). In some instances, the nodes affected were situated higher in the groin (Fig. 37), and even those of the flank might be enlarged and indurated.

Usually there were only one or two nodes involved on each side, and these were of a rounded or oval shape. Sometimes a number of small nodes were grouped together in one mass (Fig. 40); occasionally the nodes were flattened or lay in positions which made it difficult to appreciate the full extent of their enlargement. This may be seen by a comparison of the left inguinals in Figs. 41 and 42, in the latter of which the node has been picked up in order to show its actual size. This photograph also serves to convey some idea of the degree of induration in these nodes which when fully developed were extremely hard and of a semitranslucent appearance.

Significance of Lymphoid Involvement.—As will be noted by a comparison of the various figures, there was no apparent relation between the size of the scrotal lesion and the extent of the lymphadenitis. It frequently happened that the relative involvement of scrotum and nodes was in inverse proportion. The force of this statement is well brought out by Fig. 43 which shows a most pronounced lymphadenitis in an animal with comparatively slight scrotal lesions. Involvement of the inguinal glands appeared to be more an index of the activity of the scrotal infection and of the spread of the infection than of the extent of the local reaction in the scrotum.¹

¹ In addition to the observations here recorded, this statement is based upon a long series of experiments in which the presence or absence of localized infection in the lymph nodes has been determined at periods of from a few hours after inoculation to more than 2½ years. The details of this work will be reported later. It is sufficient at present to say that enlargement and induration of lymph nodes in the rabbit always signify existent infection of the nodes.

As long as there was active infection in the scrotum, the inguinal nodes were apt to remain enlarged and indurated, but as the scrotal infection subsided, the nodes usually softened and diminished in size. Occasionally these changes occurred even before any very definite change in the scrotal lesions was noted, while in other instances, the adenopathy persisted even though the scrotal lesions showed decided evidence of regression or after they had healed completely.

Enlargement and induration of the inguinal lymph nodes in the rabbit were so constantly associated with scrotal infection that they could be used as diagnostic signs when any doubt existed as to the presence or absence of specific infection in the scrotum; not only this, but these signs could be so used before a scrotal lesion had had an opportunity to develop. The chief interest in involvement of the regional lymphatics, however, is their significance in connection with dissemination or generalization of the infection.

SUMMARY AND CONCLUSIONS.

From a study of the phenomena of the primary infection on the one hand, and the phenomena of local spread, or dissemination, on the other, it is seen that a multiplicity of lesions develops in the testicle and scrotum of the rabbit which have much the same characteristics irrespective of their origin. Some of these lesions are clearly recognizable as primary lesions or parts of a primary reaction to infection, while others are just as clearly the results of dissemination of the virus from a primary focus of infection or correspond with lesions which are commonly spoken of as secondary lesions. The effort to draw a sharp line of distinction between these two groups of lesions or between a primary and a secondary stage of infection in the rabbit, however, would be largely an arbitrary procedure. The fact is that the tissues of the scrotum and testicle of the rabbit constitute favorable surroundings for the localization and development of *pallidum* infections. Under ordinary circumstances, a large part of the reaction to infection which expresses itself in the formation of lesions recognizable by ordinary methods of examination takes place in these tissues. These lesions present certain broad and general characteristics without regard to whether they are primary or secondary in origin; the reaction is merely a reaction to a syphilitic infection which in either case may assume the most diverse character.

Further, it would appear that in rabbits infected with such strains of *Treponema pallidum* as we have used, the virus is never confined to the area occupied by the so called primary lesion, or chancre, but always spreads and always gives rise to a regional adenopathy. There may be no lesions to indicate the progress of this dissemination, but an examination of the inguinal nodes shows that dissemination occurs very soon after inoculation, and a *pallidum* reaction may be detected in these glands even before infection can be recognized in the scrotum. Subsequently lesions develop in all parts of the scrotum and testicle, sometimes involving the entire testicle or scrotum, and at others, forming focalized lesions with an especial predilection for certain locations such as the epididymis, the mediastinum testis, the tunics, and the dorsal folds of the scrotum. In some instances, more or less continuous lesions form along the course of the perivascular lymphatics, suggesting that this is one path taken in the dissemination of the organism. It is probable, however, that lesions of a gross character develop more as a result of accumulation of spirochetes than of mere invasion of the lymphatics since they are not a constant accompaniment of the local infection, while invasion of the lymphatics and extension of the infection to the regional lymph nodes occur in all cases.

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EXPLANATION OF PLATES.

The illustrations are all reproductions of untouched photographs which represent the objects at their natural size. Except where otherwise indicated, the statements of time are all estimated from the date of inoculation.

PLATE 90.

FIGS. 1 to 6. Lesions resulting from local extension, or local dissemination, in the scrotum.

FIG. 1. 85 days. Diffuse involvement of the scrotum with the formation of multiple focal lesions. The primary lesions are marked by arrows.

FIG. 2. Same animal. 91 days. Subsidence of the diffuse reaction with rapid evolution of the focal lesions.

FIG. 3. Same animal. 113 days. Final stage in the transformation of the disseminated lesions into a chancre-like lesion.

FIG. 4. 56 days. Perivascular lesions extending from the outer edge of an implant about which there has been very slight reaction. The implant is marked by an arrow.

FIG. 5. 63 days. A diffuse thickening of the scrotum with perivascular infiltrations along the vessels of the dorsal fold. The seat of the original chancres is indicated by the tiny crust on the right and the marked depression on the left.

FIG. 6. Same animal. 85 days. A later stage of the same lesions.

PLATE 91.

FIG. 7. 69 days. Multiple focal lesions of the scrotum with tendency to chancre-like transformations.

PLATE 92.

FIGS. 8 to 10. Recovery of incompletely healed chancres after treatment.

FIG. 8. 40 days. The chancres at the time of treatment.

FIG. 9. 2 weeks after treatment, marking the extent of the regression produced.

FIG. 10. 4 weeks after treatment. Compare the regeneration effected with the original lesions and with the extent of the regression produced in the two chancres.

FIGS. 11 to 13. Local recurrence after complete healing of scrotal chancres.

FIG. 11. 29 days. The chancres at the time of treatment.

FIG. 12. 2 weeks after treatment. The right chancre is healed, the left almost healed.

FIG. 13. 78 days after treatment. A recurrence in the form of multiple focal lesions which bear but little resemblance to the original chancres and only one of which could be regarded as a chancre recurrence.

PLATE 93.

FIGS. 14 to 16. Recurrence of focal and diffuse lesions after healing of chancres.

FIG. 14. 43 days. The chancres at the time of treatment.

FIG. 15. 35 days after treatment. The chancres completely healed.

FIG. 16. 64 days after treatment. Recurrence of multiple nodules with diffuse infiltration of the scrotum.

FIGS. 17 to 19. Recurrent papular lesions at the edge of the scar of a healed lesion.

FIG. 17. 37 days. The chancres at the time of treatment.

FIG. 18. 21 days after treatment.

FIG. 19. 77 days after treatment. A thin, smooth scar with two small papules at its outer edge.

PLATE 94.

FIGS. 20 to 22. Healing of scrotal chancres with recurrence of chancre-like lesions entirely away from the site of the original lesions.

FIG. 20. 50 days. The original chancres.

FIG. 21. 7 days after treatment. Ulcers healed and chancres rapidly resolving.

FIG. 22. 133 days after treatment. Recurrent chancre-like lesion in the dorsal fold of the scrotum. The testicle is rotated towards the median line.

FIGS. 23 to 25. Transformation of a healing chancre into a diffuse infection of the scrotum followed later by multiple focal lesions of an erythematous character grouped mainly about the edges of the scar.

FIG. 23. 48 days. The time of treatment.

FIG. 24. 21 days after treatment. Chancres almost resolved but replaced by a diffuse infiltration of the scrotum. Note the thickened and refractile fold of the scrotum on the right.

FIG. 25. 35 days after treatment. Multiple focal lesions grouped particularly about the edges of the scars. Slight exfoliation.

PLATE 95.

FIGS. 26 to 28. Healing of scrotal chancres with recurrence in the head of the epididymis.

FIG. 26. 48 days. The chancres.

FIG. 27. 15 days after treatment. The chancres in process of healing.

FIG. 28. 30 days after treatment. The chancres healed; recurrent nodules in the head of the epididymis in both testicles.

FIGS. 29 to 31. Late recurrence of a minute papular lesion.

FIG. 29. 46 days. The chancres.

FIG. 30. 43 days after treatment. The chancres almost healed.

FIG. 31. 105 days after treatment. The recurrence. A minute point of infiltration in the scrotum, marked by the arrow.

PLATE 96.

FIGS. 32 to 37. Inguinal adenopathy associated with scrotal infection.

FIG. 32. Appearance of the inguinal region of a normal rabbit.

FIG. 33. 5 days. Acute swelling of inguinal nodes.

FIG. 34. 7 days. Acute swelling. Left, a single enlarged node; right, a large node situated over the cord and two smaller nodes at the edge of the ring, marked by arrows.

FIG. 35. 18 days. Enlargement with induration. Marked but unequal involvement of the nodes. Chancres small.

FIG. 36. 18 days. Marked lymphadenitis on the left with two small shotty nodes on the right, marked by arrows.

FIG. 37. 82 days. An asymmetrical involvement of the inguinal nodes. The enlarged node on the left is situated high in the groin.

PLATE 97.

FIGS. 38 to 43. Inguinal adenopathy associated with scrotal infection.

FIG. 38. 23 days. Lymphadenitis of moderate degree.

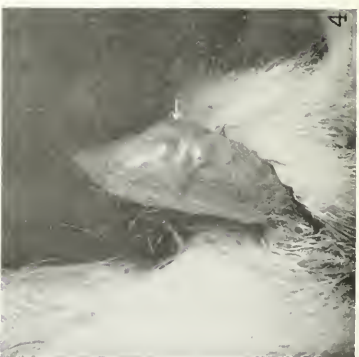
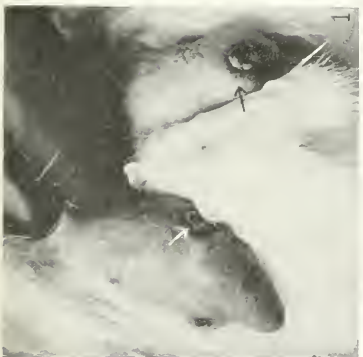
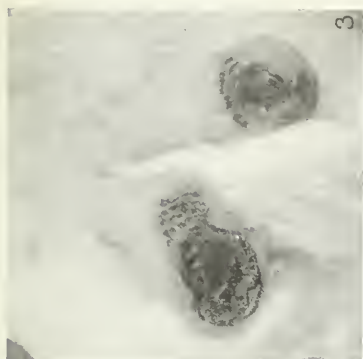
FIG. 39. 28 days. Indurated nodes. Chancres small; nodes large and unequal.

FIG. 40. 64 days. A flattened mass of small nodes on the right with a single rounded node on the left. Chancres large; nodes moderately enlarged but intensely indurated.

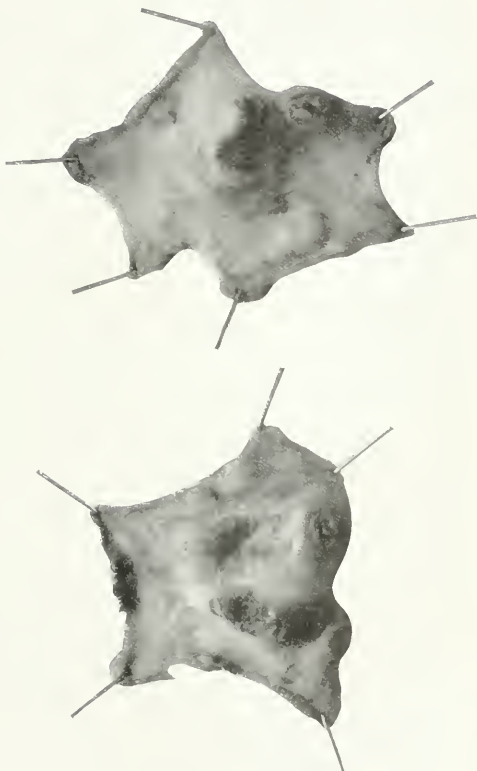
FIG. 41. 60 days. Extremely large chancres with apparently rather small lymph nodes. The nodes are deep, however, and lie directly in the crease of the groin.

FIG. 42. Same as Fig. 41. The left node picked up between the thumb and finger.

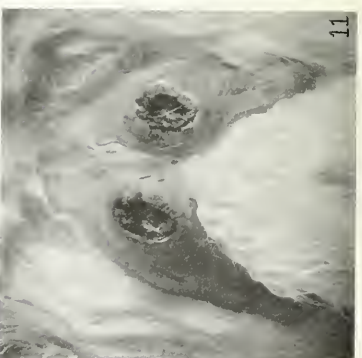
FIG. 43. 63 days. Marked equal and symmetrical lymphadenitis with slight lesions of the scrotum. Same animal as in Fig. 5.

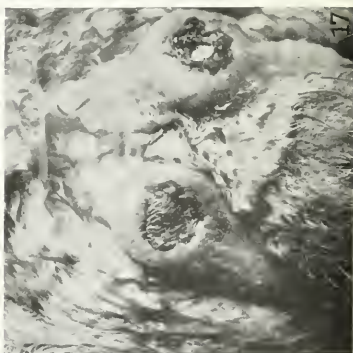
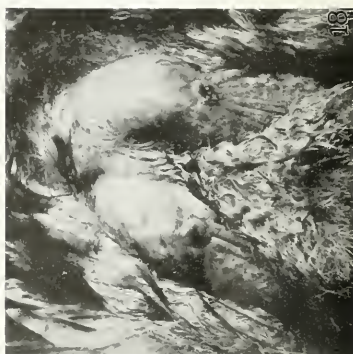
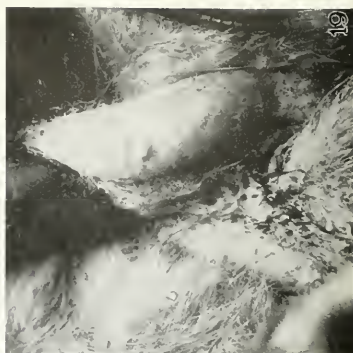


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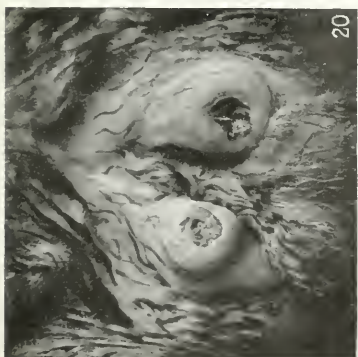
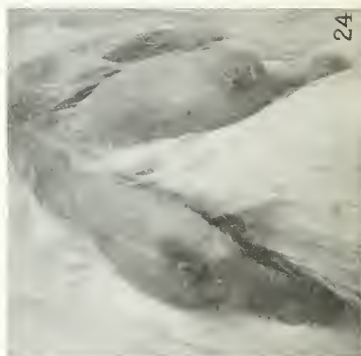
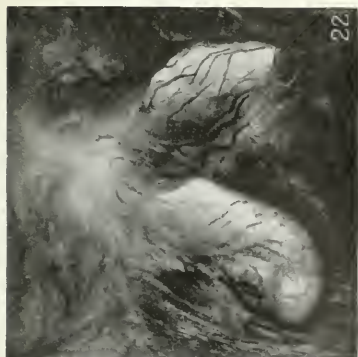


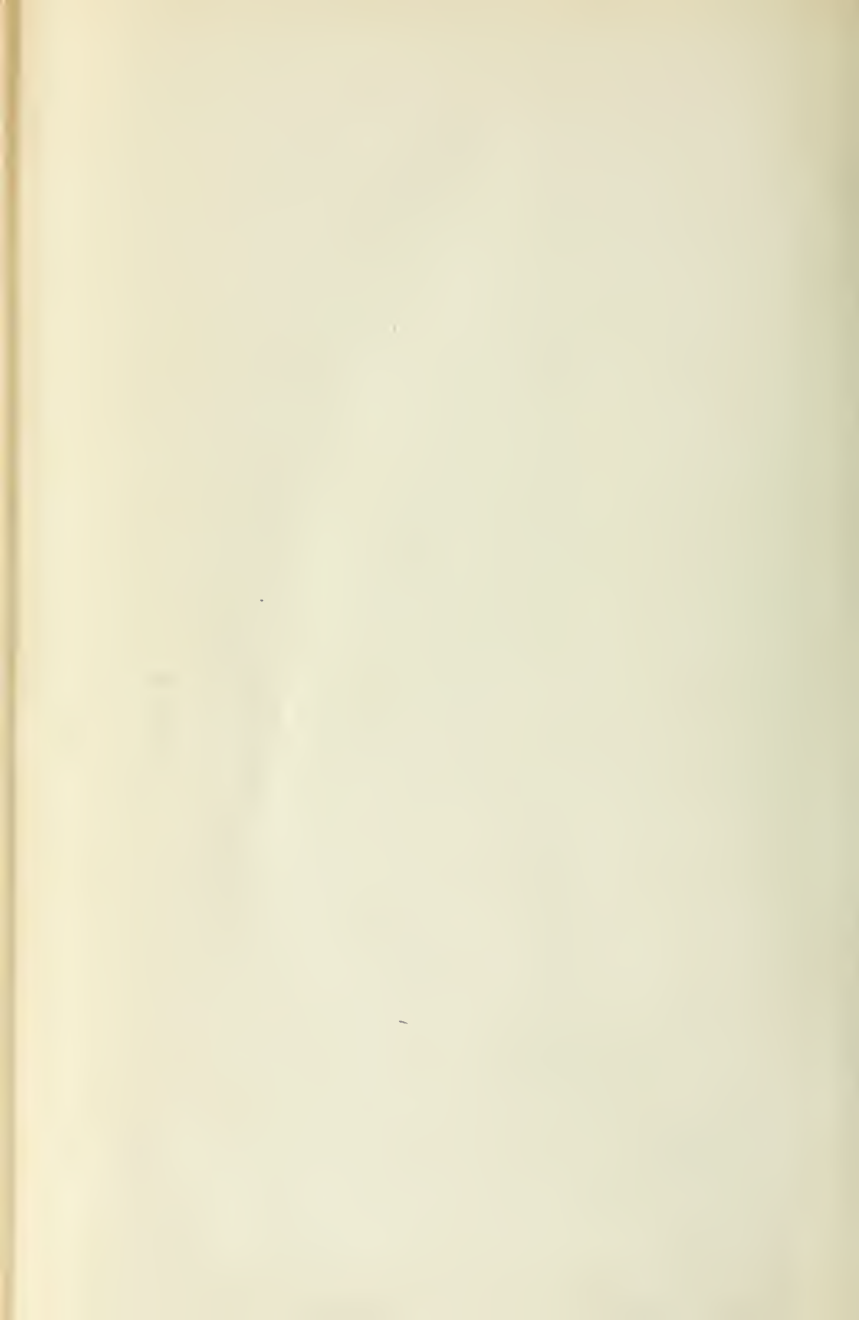


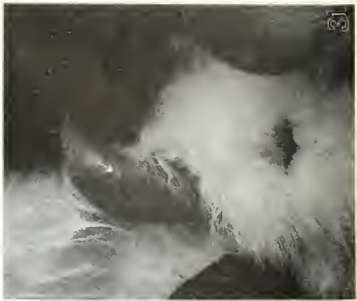
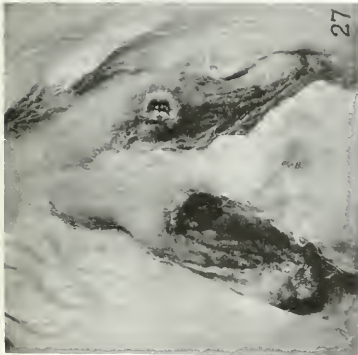




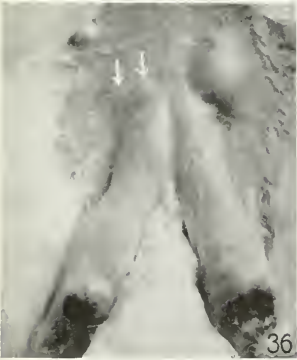


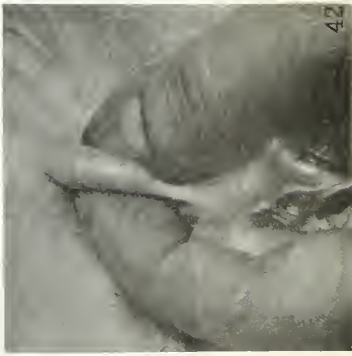
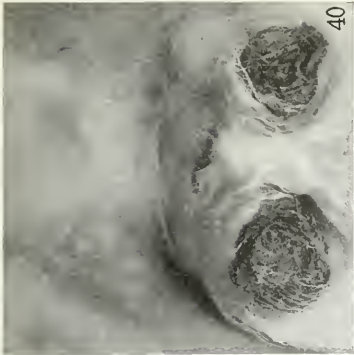












HETEROTRANSPLANTATION OF THE THYROID GLAND.

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In two preceding communications the heterotransplantation of skin of the guinea pig¹ and of the pigeon² was discussed. At that time it was thought desirable to add to these studies others in which glandular tissues, free from bacteria, were used instead of skin, which is exposed to bacterial contamination. In this paper we deal with the heterotransplantation of the thyroid in mammals. This will be followed by a study of heterotransplantation of kidney tissue.

Our aim in these investigations is twofold. In the first place we wish to find the cause of the lack of success of heterotransplantation. And in the second place we use heterotransplantation as an additional method through which to analyze the laws underlying the reactions of various kinds of tissues towards each other.

Transplantation of the Thyroid Gland of the Guinea Pig into Rats.

Lobes of thyroid of guinea pigs were transplanted into twenty-four rats. In some instances only one, in others two lobes were transferred into each rat; occasionally a lobe of thyroid was transplanted into one side and a piece of kidney into the other. All pieces were placed into pockets of the subcutaneous tissue which had been made through a dorsal incision. The results were briefly as follows:

3½ Days after Transplantation (One Experiment).—The majority of acini are necrotic, and the acinus cells are pycnotic. In some places there are some better preserved acini, but with beginning degeneration (caryolysis). There are some fairly well preserved acini, with well preserved colloid. Only one mitosis is found in the transplant, and it is doubtful whether this mitosis is in an acinus cell.

¹ Loeb, Leo, and Addison, W. H. F., *Arch. Entwicklungsmechn. Organ.*, 1909, xxvii, 73.

² Loeb, Leo, and Addison, W. H. F., *Arch. Entwicklungsmechn. Organ.*, 1911, xxxiii, 44.

Between and around the best acini a limited number of fibroblasts, some of which are hypertrophic, are found growing. But on the whole very little connective tissue grows between acini and into the necrotic center. In some places connective tissue cells grow between acini into the center without noticeable recovery of nearby degenerating acini. Few lymphocytes are visible, but in places connective tissue cells and some lymphocytes penetrate into the better preserved acini and may destroy some of them. No vessels are visible between the acini.

After 4 Days.—Many acini are relatively well preserved and a number of mitoses are seen in acinus cells. The well preserved acini are found where the surrounding connective tissue is more active and where some fibroblasts grow around and between acini. On the whole, few fibroblasts move between acini. Where the connective tissue is not active in the periphery of the transplant, the acini show somewhat shrunken nuclei. The center is necrotic, and little connective tissue is growing into it. In the periphery of the necrotic part some vessels are still preserved.

After 5 Days.—Several grafts were examined; they did not differ much from each other. The majority of the acini are not well preserved, but shrunken or in a state of caryorrhexis. In places there are better preserved acini, especially where a few better preserved fibroblasts are seen. The outer part of the parathyroid is preserved, the inner part necrotic. The connective tissue shows little activity. Only few fibroblasts grow around and between acini; they have a tendency to form fibrous tissue. Few vessels are near the transplant, but in certain places a few capillaries are found near by. Even directly around the capillaries the fibroblastic tissue becomes transformed into fibrous tissue. There are only few lymphocytes near acini, but in some places where vessels are around the transplant, there are many lymphocytes which may infiltrate some acini. This is, however, a comparatively rare finding. Lymphocytes are more frequently found where acini are missing. The center is necrotic. Some fibroblasts grow into the center and often degenerate here. Phagocytes take up some necrotic material.

After 6 Days.—Examination of several transplants showed essentially the same condition as in the previous stage. The number of well preserved acini is small. Their situation shows a relation to fibroblastic activity. In most places acini are shrunken and their cells vacuolar and caryolytic. There are in most pieces a few mitoses present in acinus cells, and some acini contain colloid. The connective tissue shows on the whole little activity. Where it does grow between acini, it has a tendency to form fibrous tissue. Only a small part of the central necrotic material is organized. Some capillaries grow around the transplant, and only rarely do they grow a short distance between some acini. In some places the lymphocytic infiltration in the neighborhood of the transplant is considerable, in others it is slight. Occasionally the number of lymphocytes around some acini is greater, and here they may even penetrate into the interior of some of the latter. This is found especially where a number of vessels are in the direct

neighborhood of the acini. Sometimes a few polynuclear leucocytes are seen in the transplant. In one animal the transplant has been entirely destroyed.

After 7 and 8 Days.—The transplants were found to be destroyed. Only fibrous tissue, some necrotic material, and lymphocytic infiltration were seen.

After 9 Days.—In three animals the transplants had been destroyed. A great part of the necrotic material had been organized. Some lymphocytic infiltration was visible. In a fourth and fifth rat some of the transplants were still alive. In the fourth animal there were still better preserved acini with a number of mitoses and large vesicular nuclei on one side of the transplant; but even here cells were somewhat vacuolar. A few, but not many connective tissue cells and lymphocytes were noted around the acini. In some acini retracted colloid was visible. In proliferating acini desquamating acinus cells had gotten into the colloid. In some acini (or remnants of ducts(?)) several rows of cells were formed. On the other side of the transplant acini were very vacuolar and degenerating; there was here no proliferation of connective tissue cells, and lymphocytes were absent. Especially noticeable is the absence of blood vessels between acini and in the center. The necrotic center is incompletely organized. In the fifth animal a small number of acini is still preserved. Some mitoses and some hypertrophic nuclei are seen in acinus cells. Dense fibrous tissue with few vessels compresses these acini; only few fibroblasts are between the acini. Some lymphocytes are around acini, and a few of these have gotten into the acini. There is no dense lymphocytic infiltration directly around the acini. Where on the other side a dense lymphocytic infiltration is present, acini are found to be lacking. The effect of compression by connective tissue is here more in evidence than the lymphocytic activity. Vessels are very scarce or absent between preserved acini.

9 days after transplantation was the latest date at which living thyroid tissue was still found. Following this period only connective tissue with or without some necrotic material and lymphocytes were observed. In some pieces infection had evidently taken place.

Conclusions.—As late as 9 days after transplantation a number of acini are preserved in two pieces and mitoses occur at this time. This is the latest term at which mitoses and living tissue are found. In general the greater part of the heterotransplanted thyroid suffered largely through the lack of suitability of the body fluids of the host. Many of the acini were degenerating in various ways. Evidently only those situated under especially favorable conditions were found better preserved. Wherever the acini were in a better condition the fibroblasts of the host showed greater activity near by; and conversely, where they were in a poor condition the fibroblasts were relatively inactive. But on the whole, the number of fibroblasts growing between the acini was relatively small, and the fibroblasts which did

grow around the acini had a tendency to form fibrous tissue. The blood vessel supply around and between the acini was very poor; but some capillaries penetrated here and there a little between some acini.

The lymphocytes were usually not numerous between and in the direct neighborhood of the acini. But in some cases they were more frequent at some distance from the transplant. Occasionally they extended from here into the transplant itself and invaded a few acini. Thus they did to a very limited extent contribute to the destruction of acini.

We see then that it is primarily an incompatibility between the body fluids and the transplant which is responsible for the small amount of surviving tissue. The destructive action of the lymphocytes and connective tissue plays only a minor part. The lymphocytes invade the transplant to a very slight extent. Vascularization and ingrowth of fibroblasts into the transplant are slight, much less so than in homoiotransplantation. In the latter there is also much more lymphocytic infiltration. Furthermore, the transplanted tissue is usually much better preserved and more active in homoiotransplantation.

Transplantation of the Thyroid Gland of the Rabbit into Rats.

After 3 Days.—The ring of acini is incomplete; it is interrupted, where muscle of the host forms the base of the graft. Elsewhere are well preserved acini with colloid. The acinus cells are succulent and seven to nine mitoses can be seen in acinus cells. Pycnotic nuclei disappear only in the periphery of the necrotic area. Fibroblasts move between the acini; some mitoses are visible in fibroblasts. Polynuclear leucocytes are found only in one place.

After 5 Days.—A well preserved ring of acini with retracted or vacuolar colloid is found. The cells of the acini are in good condition; they have on the whole a succulent character and the nuclei are well formed. Mitoses are, however, very rare in the acinus cells. Some fibroblasts and capillaries grow between the acini; but in general the amount of fibroblastic tissue proliferating around the acini is small. In one place, however, the fibroblasts are active and near by a mitosis is found in an acinus cell. When the acini are best preserved, most fibroblasts grow around them; here they make fibrous stroma. The center is not yet organized. Large cavities originate, probably through solution of necrotic tissue. In the connective tissue capsule there are lymphocytes which do not penetrate into the acinar zone. Polynuclear leucocytes are absent.

After 7 Days.—There is a well preserved ring of acini. Five mitoses were counted in acinus cells. The latter are of medium size and show vesicular nuclei. Some of the colloid is in good condition, other colloid is rarified or vacuolar. There are some cells in the colloid. Fibroblasts and a few capillaries penetrate between the acini. Only the peripheral part of the necrotic center is organized by a small amount of connective tissue. The zone of acini is surrounded by a fibrous capsule which is somewhat infiltrated with lymphocytes. The capsule with the lymphocytes is rather sharply separated from the thyroid ring. Few lymphocytes penetrate between acini and a very small number of polynuclear leucocytes is visible.

After 8 Days.—In this specimen conditions are in many respects similar to those in the preceding specimen, but there are certain differences. There are many well preserved acini, some of which are close together, and some mitoses are found in the acinus cells. The center is necrotic; here there is much infiltration with polynuclear leucocytes. These cells cause cavity formation in the peripheral parts of the necrotic material. A small part of the necrotic material is organized. Connective tissue surrounds and invades some acini; the fibroblasts form fibrillar tissue. Lymphocytes accompany the connective tissue, and both destroy some acini, the fibrous tissue compressing the acini. Very few vessels are visible and they enter the tissue mainly at places where thyroid acini are missing. Because of the lack of vessels which penetrate between acini, the lymphocytes attack the acini mainly from the outside, wherever such an attack occurs. In places connective tissue may grow around some acini without being associated with much lymphocytic infiltration; the fibroblasts produce fibrous tissue. The thyroid itself is not markedly infiltrated with lymphocytes. A connective tissue capsule surrounds the transplant.

After 9 Days.—There are well preserved acini close together with only exceptional mitoses. Some well preserved colloid is present in acini. The center is necrotic; it is only partly organized by fibrous tissue. Capillaries are found around the transplant, usually at some distance from the thyroid tissue. Here and there in the capsule may be large masses of lymphocytes; but in other places in the surrounding tissue lymphocytes are lacking around the capillaries. Lymphocytes enter the thyroid only exceptionally, but in a few cases they as well as fibroblasts destroy some acini. Although some capillaries enter the thyroid proper, still there is neither much growth of thyroid acini nor much lymphocytic infiltration in the transplant proper. Through a defect in the thyroid some vessels penetrate towards the center. Usually capillaries do not break through directly between the acini. Some fibroblasts penetrate between the acini and make fibrils; they produce fibrous tissue even in the direct neighborhood of the capillaries. The capsule around the graft is fairly fibrous.

After 11 Days.—In neither of the specimens of the first rat was living thyroid tissue present. In the first specimen some necrotic material as well as fibrous and fibroblastic tissue with capillaries and some lymphocytes were found. In the second specimen an epidermal cyst or its remnants were seen. In places connec-

tive tissue was growing into the keratin and formed epithelioid cells around the particles of keratin. In certain areas polynuclear leucocytes separated the epidermis from the underlying connective tissue; in such places the epidermis was necrotic. In the periphery at a distance from the transplant there was a ring of lymphocytes. This epidermal cyst originated probably in a thyroid duct, such as was found to occur in the thyroid in the other specimens.

In a second rat some thyroid was preserved after 11 days. There were groups of small acini surrounded as a whole and individually by band-like fibrous tissue. No mitoses are found in acinus cells, the latter of which are on the whole low or cuboidal; some remnants of vacuolar colloid are present in the lumen of the acini. No large masses of lymphocytes invade them. There are small collections of lymphocytes separated from acini by fibrous bands. In places, however, some isolated lymphocytes infiltrate acini and help to destroy them. Some fibroblasts may likewise invade and destroy a few acini. Certain acinus cells are becoming vacuolar and degenerate. In the surrounding fibrous tissue quite a number of scattered polynuclears are visible which occasionally may infiltrate the acini and help to destroy them. In the fibrous tissue there is a large duct with stratified epithelium. Some epithelial cells desquamate, and some polynuclear leucocytes penetrate into the duct lumen. From this duct strands of epithelial tissue radiate; they are surrounded by fibrous tissue but are not usually invaded by lymphocytes or polynuclear leucocytes. Occasionally, however, some lymphocytes or polynuclears invade them and some of the former may also invade the stratified duct. Exceptionally, some lymphocytes around these cell strands may destroy part of them. The epithelial cells are evidently not resistant; they are near degeneration and fall an easy prey to various kinds of attacking cells such as polynuclear leucocytes, lymphocytes, and fibroblasts.

Around the ring of acini there is a concentric capsule of fibrous tissue. At the outside of the fibrous tissue we find collections of lymphocytes and some polynuclear leucocytes. These peripheral masses of lymphocytes are situated around capillaries. In the fibrillar tissue which surrounds the acini there are some capillaries, but usually no lymphocytes. Some parts of the central necrotic tissue are organized, other parts are as yet in process of organization. Two stages are found in the organization of necrotic material: (1) a hyaline substance is produced through mixture of the cytoplasm of the advancing fibroblasts and necrotic material; and (2) a fibroblastic tissue with many capillaries and sometimes lymphocytes is secondarily produced.

After 12 Days.—Here again merely stratified epithelium is found which is partly alive, partly necrotic. In the surrounding fibrous tissue there is much lymphocytic infiltration.

After 13, 14, and 15 Days.—No living thyroid tissue was found, but only some remnants of necrotic tissue, fibrous tissue, vessels, and lymphocytes. The same two strata of organizing material are observed; namely, (1) a lamellated, hyaline, avascular zone, and (2) infiltrating this a cellular, vascular, lymphocytic tissue.

Conclusions.—Living acini were found as late as 11 days after transplantation. This occurred in one of three pieces. Mitoses were found not later than 9 days after transplantation; but at that time they were exceptional. At 11 and 12 days epidermal ducts or cysts were still seen in cases in which the acini had already perished. In one instance, 11 days after transplantation, the epidermal duct had given origin to strands of squamous epithelium. This indicates that the squamous epithelium is more resistant to the injurious action of heterotoxins than the thyroid acini. Similarly we found previously that the squamous epithelium of the kidney pelvis may be more resistant to homoio toxins than the gland tissue.

Under the influence of the heterotoxins the epithelial cells degenerate directly, and in addition, they are less resistant to the action of lymphocytes, polynuclear leucocytes, and fibroblasts. They are easily affected by various kinds of injurious influences. While fibroblasts now and then penetrate between the acini, there is, on the whole, not much fibroblastic ingrowth into the thyroid; the fibroblasts however, that do grow around the acini have a tendency to form fibrous tissue. Capillaries may grow actively in the tissue which surrounds the transplant, but they do not usually grow between the acini, although in places an ingrowth of capillaries occurs. Lymphocytes may be present in large masses around the transplant near the vessels, but only here and there do they penetrate between the acini, and they then help to destroy some of the latter. Even in the neighborhood of capillaries which grow close to, or in the interstices between acini the lymphocytes are scarce. The presence of capillaries does not prevent the formation of fibrous tissue from fibroblasts; it occurs even in the direct neighborhood of the vessels.

The thyroid of the rabbit transplanted into the rat is well preserved at a relatively late stage, namely 9 days after transplantation; accordingly, mitoses are more frequent up to the 9th day than in the other kinds of heterotransplantation; but at 11 days some degenerative processes have become noticeable.

Transplantation of the Thyroid Gland of the Rabbit into Guinea Pigs.

After 3 Days.—An excellent ring of living thyroid preserved. Very rare mitoses in acinus cells. Some well formed colloid in acini. No degeneration of cells. Center necrotic. In the center of the necrotic area pycnotic nuclei are preserved, while in the periphery they have been lost. There are a few scattered polynuclear leucocytes. Some fibroblasts and a few capillaries growing between thyroid acini. Some parathyroid and transplanted fat tissue preserved.

After 6 Days.—Ring of thyroid tissue here and there preserved, but on the whole only a small part is still alive and this is not in good condition. The cells do not stain well or the tissue shows signs of shrinking. The cells are not succulent and only exceptional mitoses are seen. Some acini have colloid, others while close together are without lumen or colloid. In places capillaries enter between the acini. Some connective tissue, a few lymphocytes, and a relatively considerable number of scattered polynuclear leucocytes grow into the transplant and help to destroy it. Small celled connective tissue penetrates into the poorly preserved part, compresses it, and penetrates occasionally into the acini. Lymphocytes approach the acini from the neighborhood of the vessels and enter and destroy some acini. The number of polynuclear leucocytes between the acini is relatively large. In some places masses of polynuclear leucocytes destroy the tissue. The necrotic center is in process of organization by connective tissue. The transplanted thyroid tissue is not vigorous, but near the point of death; migrating cells contribute to this destruction.

After 7 Days.—The thyroid is necrotic. In the center pycnotic nuclei are preserved, in the periphery they are lost. Connective tissue is growing in the periphery of the necrotic piece. There is no lymphocytic infiltration.

After 8 Days.—Well preserved acini, close together with and without colloid; also larger ducts present; no mitoses. In certain areas of the thyroid ring are large acini with well preserved cells and nuclei.

A thick capsule of dense fibrous tissue with few vessels around the thyroid. Where there is a defect in the thyroid ring, many capillaries enter the transplant together with connective tissue; where the thyroid ring is preserved, only few vessels can be found. On the whole, the vascularization of the thyroid is very poor. Some connective tissue penetrates between acini, and fibroblasts form here a thin stroma; they do not usually destroy acini. Only small parts of the necrotic material in the center of the transplant are organized. In the center of the necrotic material there are still some pycnotic nuclei, while in the periphery they have been destroyed. Polynuclear leucocytes collect in the surrounding capsule; they penetrate into the thyroid and destroy some of the acini. They also migrate into the necrotic center and dissolve a part of the necrotic material. There are some lymphocytes in the surrounding connective tissue, but there is not much lymphocytic infiltration in and around acini. There are large areas of preserved thyroid without lymphocytic infiltration. In certain places, however, lymphocytes are so numerous that they destroy acini.

After 10, 11, and 12 Days.—The thyroid was no longer preserved. There was some necrotic material with some polynuclear leucocytes. There is slight lymphocytic infiltration. In one specimen parts of the necrotic material have become dissolved.

Conclusions.—After transplantation of the thyroid of the rabbit into guinea pigs the results were not so satisfactory as after transplantation into rats. Well preserved thyroid was found as late as 8 days after transplantation, but not at later dates. The last mitoses were observed 6 days after transplantation and at that time they were few in number. Otherwise the conditions are similar to those generally seen in heterotransplantations. The ingrowth of vessels is slight. Fibroblasts likewise do not grow in very actively and have a tendency to produce fibrous tissue. The capsule surrounding the graft is very fibrous. Here lymphocytes are in places present in large numbers; but they penetrate into the thyroid only to a limited extent. The distribution of the lymphocytes in the surrounding tissue depends upon the vessels. Lymphocytes and fibroblasts may here and there contribute to the destruction of acini. These transplantations differ from those in the rat in the greater number of polynuclear leucocytes which are usually found in the guinea pig. They may be seen scattered in the surrounding capsule, and from here they penetrate into the thyroid proper and help to destroy some acini. They also may enter necrotic material and dissolve some parts of it. We are inclined to assume that the inferiority of the results in this series is due to the activity of the polynuclear leucocytes or to the conditions which call forth their appearance. This difference between transplantation into the rat and guinea pig may be caused by the fact that the transplantation of tissue into dorsal pockets in the rat insures sterility of the tissue to a greater extent than transplantation into ventral pockets as practised in the guinea pig. It is possible that after heterotransplantation the transplants offer better soil for the action of microorganisms than after autotransplantation or homoiotransplantation.

Transplantation of the Thyroid Gland of the Cat into Rats.

After 3 Days.—Well preserved thyroid ring. The acini are close together and have colloid. There are medium sized cells in good condition, but some cells are slightly vacuolar. No mitoses. Fibroblasts and a number of capillaries coming

from capillaries in the surrounding tissue grow between acini. No lymphocytes. There is little organization in the necrotic center. The central part of the necrotic material is with, the periphery without pycnotic nuclei. On the whole, the piece is well preserved but the acinus cells are not succulent. In places a few polynuclear leucocytes migrate through the thyroid ring into the necrotic center.

After 4 Days.—Well preserved ring of thyroid with apparently healthy, but not succulent cells; occasionally vacuolar cells. Some acini with retracted colloid, many acini with very small lumen and little colloid. No mitoses. The central part of the necrotic material is with, the periphery without pycnotic nuclei. A few fibroblasts grow between the acini and a small number of polynuclear leucocytes wanders through the thyroid ring into the center. Occasional small collections of lymphocytes around peripheral vessels, but no marked lymphocytic infiltration of the transplant. Blood vessels and probably also lymph vessels grow from the outside into the center through the thyroid; they probably are connected with the old preexisting vessels in the graft. Even in the neighborhood of the blood vessels no mitoses in acinus cells.

After 5 Days.—Well preserved ring of thyroid; acini with colloid. Only exceptional mitoses in acinus cells; even where there is much fibroblastic tissue around acini, there are usually no mitoses. No succulent acinus cells. In the fibroblastic tissue which surrounds the transplant many blood vessels are seen and occasionally some collections of lymphocytes around vessels. A vessel grows from the outside into septa of the thyroid; in this case probably a transplanted vessel connects with neighboring vessels. Where there is much fibroblastic tissue around the transplant, fibroblasts and some vessels may grow around acini. Fibroblasts with some lymphocytes may separate, and occasionally penetrate into acini. Usually lymphocytes are absent around acini. In the periphery of the piece we find cavities; they are either lymph spaces or dissolved necrotic tissue.

After 6 Days.—There is not so much thyroid tissue preserved as in some of the previous specimens, but on one side of the piece a zone of acini is present. In some acini there is retracted colloid, in others it is lost. Mitoses are lacking. Around the transplant there is fibroblastic tissue with many capillaries. Where there is much fibroblastic growth in the neighborhood of the acini, some vessels, fibroblasts, and lymphocytes penetrate between, and some lymphocytes penetrate even into the acini. Fibroblasts provide a stroma between some acini. On the whole, there are few lymphocytes around or in the thyroid. Only isolated connective tissue strands are growing into the necrotic center. In places there are large spaces around the thyroid.

After 7 Days.—Well preserved but incomplete thyroid ring. Acini close together and with colloid which is somewhat vacuolar. The nuclei of the acinus cells are often a little deformed, and some vacuoles are found around them. Fibroblasts and capillaries proliferate around grafts. In places capillaries grow plentifully from the outside in between acini; yet mitoses in acinus cells are absent and there is not much lymphocytic infiltration around acini. Fibroblasts fur-

nish some stroma for the acini, growing between them. Here some mitoses in fibroblasts may be found. In the surrounding fibroblastic tissue there is in places a moderate infiltration with lymphocytes. Lymphocytes may also penetrate between acini and destroy a few of them, but the infiltration is not extensive, being much less marked than after homoioplastic transplantation. Some polynuclear leucocytes are in the necrotic material where thyroid is lacking.

After 8 Days.—An incomplete ring of fairly well preserved acini with colloid. Some acinus cells are vacuolar and the nuclei irregular; in general acini healthy but not turgescient. The acini form islands in the periphery of the graft. Where fibroblasts and vessels are found near the thyroid, they may penetrate into the transplant. Connective tissue furnishes a stroma for the thyroid; while it has organized some necrotic material, most of the latter is as yet unorganized. In places connective tissue cells and a few lymphocytes penetrate between and into acini and destroy some, but not many of them. In such places we may find some mitoses which are probably of fibroblastic origin. There is no real lymphocytic infiltration. Some large lymph vessels grow into the center of the thyroid and adjoin directly the living thyroid. Notwithstanding this fact, there is not much lymphocytic infiltration. Some parathyroid is also preserved.

After 9 Days.—The results obtained with two pieces are similar. There is an incomplete ring of thyroid tissue. The acini are in many places well preserved and may contain retracted colloid or be without lumen; many acini are close together. In other places, especially where fibrous tissue surrounds and compresses the acinus cells, some of the latter are vacuolar and the nuclei deformed. There are some, but not many mitoses in acinus cells. Where fibroblasts penetrate into acini and destroy them, mitoses are found, but whether these are in acinus cells or fibroblasts is uncertain. A few capillaries are found around acini, but the presence of vessels does not call forth cell proliferation. On the whole, the connective tissue proliferation between the acini is not marked. From the surrounding connective tissue capsule connective tissue grows between acini, surrounds some of them, and chokes them, thus contributing to their destruction. These acini may disintegrate. In other places fibroblasts proliferate around acini; here the latter are relatively better preserved. But fibroblasts may also penetrate into some acini and destroy them. There is no noticeable lymphocytic infiltration. Lymphocytes are found in the surrounding capsule and with the fibroblastic tissue they may penetrate between some acini and help to destroy some of them. However, they do not play a significant part and are less important than the connective tissue so far as the destruction of acini is concerned; but even the connective tissue is not very active as a destructive agent. The necrotic center is only partly organized. Connective tissue penetrates into it, especially where the thyroid ring is interrupted. Through solution of the necrotic material cavities are formed which are not unlike lymph spaces.

At one place in the connective tissue, where the thyroid ring is interrupted, squamous cell ducts are found in the connective tissue; structures not unlike keratin pearls are formed from these ducts.

After 10 Days.—Small areas of acini are left; some acini are close together; there is still colloid present in some of them. On the whole, acinus cells are not well preserved; there is much vacuolation, especially where fibrous tissue compresses acini. Usually living acini prevent connective tissue from organizing necrotic material. Elsewhere connective tissue grows into the necrotic center which has as yet been only partly organized. Strands of fibroblastic-fibrillar tissue with some lymphocytes get near the thyroid acini, but usually stop here. Occasionally, however, connective tissue and some lymphocytes penetrate into acini and help to destroy them, but this is not a frequent occurrence. Where fibroblasts and some lymphocytes invade acini, mitoses are found, perhaps in acinus cells. Wherever there is fibroblastic proliferation near the thyroid, some lymphocytes may penetrate into adjoining acini and help to destroy them. In some places in the surrounding connective tissue there is much lymphocytic infiltration. The connective tissue growing around acini forms occasionally fibrous bands which may choke acini which thus become vacuolar. In another animal the findings are similar, but the acini are at least in part fairly well preserved; there are numerous acini with some colloid. A few mitoses and hypertrophic nuclei occur in acini. In other places, however, the acini become vacuolar and their nuclei pycnotic. Some acini are close together; others are surrounded by fibrous tissue. There is no dense lymphocytic infiltration in, or directly around the acini, but a few lymphocytes and connective tissue cells seem to migrate into some acini. The connective tissue around the acini is fibrous or fibrillar. In places such fibrillar-fibroblastic tissue encircles acini and penetrates between them. Some fibroblasts grow around the better preserved acini, but usually the connective tissue around the acini is fibrous. In such acini degenerative processes may set in. Whole acini may become pycnotic; other cells become vacuolar. Migration of lymphocytes and small connective tissue cells, if it takes place at all, is isolated; in the surrounding fibrous capsule, on the other hand, the lymphocytic infiltration may be considerable. There are a few mitoses in acinus cells. On the whole, the vascularization is very poor; in places, however, there is vascular tissue with many capillaries around the thyroid, and here may be found some lymphocytes. At one place a vessel with some lymphocytes penetrates between the acini. There are frequent mitoses either in fibroblasts or endothelium of blood vessels and the connective tissue penetrates into the central necrotic tissue especially where the thyroid ring is interrupted. The tissue supplanting the necrotic material is dense.

After 11 Days.—Peripheral thyroid tissue is preserved, but many acinus cells show signs of degeneration, especially vacuolation. Acini situated in fibrous bands may become transformed into small cell nests without colloid. Other acini, however, still retain some colloid. A few mitoses are observed in acinus cells, but their number is smaller than in homoiotransplants at the corresponding stage. The stroma is usually fibrillar or fibrous and shows very poor vascularization. Connective tissue cells frequently break into the ring of acini and into the acini themselves and destroy them. Also a few, but not many lymphocytes may

penetrate between and into acini. Usually the connective tissue forms fibrils or fibrous bands around acini, thus choking them. The connective tissue penetrates also through the ring of acini to the inner aspect of it and attacks it from the inside. Here again it forms fibrous tissue. The thyroid ring exerts a certain, though slight restraining influence on the connective tissue. Thus the thyroid to a certain extent inhibits the rapid organization of the central necrotic material. This organization proceeds more rapidly where the thyroid ring is absent, because here connective tissue can penetrate more readily into the necrotic material. Thus it comes about that the central necrotic material may adjoin directly the thyroid ring. Under the conditions of heterotransplantation the thyroid still has some restraining influence on the connective tissue, but it is very much restricted. Furthermore, the epithelial elements under the influence of the heterotoxins show little power of resistance to injury. The constricting effect of the fibrous tissue, the absence of good vascularization, and the direct attacks by fibroblasts and lymphocytes, though limited, all contribute to the destruction of the graft. These destructive factors are not counterbalanced by a new production of tissue sufficient to make good the constant loss. In the necrotic material polynuclear leucocytes produce in certain places a solution of the material.

After 12 Days.—In the first animal only little thyroid tissue is preserved; considerable parts of the thyroid are degenerating. Peripheral acini become vacuolar and the stroma between them also degenerates in places. There are visible areas of collapsed acini without colloid. The degenerating parts of thyroid are replaced by fibrous tissue. Some connective tissue cells and lymphocytes migrate into the degenerating areas. Fibrous tissue, poor in cells, surrounds acini. Only little lymphocytic infiltration. There are, however, still small bundles of acini infiltrated by some lymphocytes; there is in places much lymphocytic infiltration in fibrous tissue around and at some distance from the thyroid. Here the tissue may be rich in fibroblasts and blood vessels. Much necrotic tissue is organized, especially where the thyroid is interrupted. The organization takes place in two stages. In the first stage much necrotic tissue is organized by avascular connective tissue; secondarily this tissue becomes organized by fibroblastic, lymphocytic tissue with blood vessels. At one place in the necrotic material there is an abscess (localized infection). In another animal no living thyroid was found in two pieces, only necrotic material, fibrous tissue, and some lymphocytic infiltration.

After 14 Days.—Conditions are similar. Many acini are in process of degeneration; they show vacuolation or are surrounded by dense fibrous tissue and are small, evidently compressed, and without lumen and colloid. In other acini colloid is found. Mitoses in acinus cells are absent. Vascularization is poor, but a few capillaries are found between acini. The connective tissue has a tendency to form dense fibrous encircling bands around acini, but in some places it is still richer in cells and less fibrous, and thus it penetrates between acini. Such connective tissue pushes into some acini and may destroy them. Wherever the connective tissue is still more fibroblastic, some lymphocytes are usually found

in it which may enter acini and contribute to their destruction. But on the whole the lymphocytic infiltration is slight, and even where lymphocytes are near the thyroid, they usually do not enter the thyroid proper; they do not seem to be attracted by the heterologous tissue. The fibroblastic character of the connective tissue is present only in certain places, and only occasionally is a mitosis found in a fibroblast between acini. Soon this connective tissue becomes converted into fibrous tissue very poor in cells. Connective tissue penetrates also into the necrotic center, mainly at places where the thyroid ring is interrupted. The thyroid acini present a certain obstacle to the advance of the connective tissue. The necrotic tissue is therefore least organized where it adjoins directly the thyroid. The organization of the necrotic tissue takes place in two phases. First a hyaline avascular tissue is produced which is later substituted by fibroblastic vascular tissue.

In a second animal a greater part of the transplant was dead 14 days after transplantation. The majority of the acini which have not yet become necrotic are dying. They are vacuolar and the nuclei are deformed or have disappeared. There are still some acini with vesicular nuclei. Where all the tissue is necrotic the peripheral parts are organized by connective tissue and blood vessels. Near such blood vessels there are considerable masses of lymphocytes. These lymphocytes, however, do not usually penetrate into the living, vacuolar thyroid tissue. The acini are often surrounded by avascular fibrous tissue. A few connective tissue cells may penetrate between the dying transplanted acini and surround some of them. Around the transplant there are in places some empty spaces, probably the result of the solution of necrotic material. Such spaces were also found in the second animal at this period.

The tissue is injured primarily as a direct result of heterotoxin action. The behavior of fibroblasts and blood vessels and to some extent the action of lymphocytes contribute to the destructive result.

After 15 Days.—No living thyroid left. A small amount of unorganized necrotic material was still present. The greater part of it had been changed into fibrous tissue around which there was much lymphocytic infiltration. In the necrotic material there were some giant cells.

After 16 Days.—No living thyroid found. In the periphery there are some necrotic acini with shrunken cells and nuclei. A part of the peripheral necrotic tissue is organized and substituted by fibrous tissue. The graft is surrounded by dense fibrous tissue with very few vessels. Here there is in places marked lymphocytic infiltration. In the necrotic transplant itself no lymphocytes are found. Some empty spaces are seen, similar to those described above. They may represent lymph spaces or dissolved fat tissue. There are some epithelioid and small giant cells around the necrotic part. In the fat disintegrating polynuclear leucocytes are found.

After 18 Days.—The transplant has become entirely necrotic with the exception of a few very vacuolar acini in the periphery. The nuclei in these acini are mostly deformed. There is no marked infiltration by lymphocytes or connective tissue

cells. No blood vessels can be seen between the acini which are still left in the periphery. A part of the necrotic material has been organized by connective tissue. As usual the organization takes place in two stages. At first a hyaline tissue is formed in which vessels are lacking. It represents a substitution of necrotic material by fibroplasm or a mixture of the two. Later a cellular connective tissue with lymphocytes supplants it. Around the graft there is dense fibrous tissue with many lymphocytes.

Conclusions.—At an early stage following transplantation the thyroid tissue of the cat is fairly well preserved and colloid may be found in the acini; but at no time does the transplanted parenchyma have a succulent character, such as may be observed after autotransplantation or even after homoiotransplantation. As early as 5 days after transplantation an occasional invasion of an acinus by fibroblasts and lymphocytes and a subsequent destruction of these acini are found. These conditions prevailed from the 3rd to the 7th day after transplantation. After 7 days some signs of beginning degeneration appear in some acinus cells, such cells being vacuolar. Many acini are, however, well preserved. At 8 days vacuolation is also noticeable. At 9 days degeneration is distinct in some places. Fibrous tissue surrounds and compresses some acini. Other acini are destroyed by invading fibroblasts and lymphocytes. This is, however, not a prominent feature of the process. After 10 and 11 days the destructive effect of compression by fibrous tissue is still more marked. Whole acini become vacuolar or even pycnotic. On the whole, conditions are similar to those found after 9 days, but perhaps more marked. After 12 days there is much degeneration of the thyroid. The amount of preserved tissue is relatively small. Vacuolation and collapse of acini are noticeable. After 14 days the same vacuolation and compression of acini are found. There is much degeneration. Fibroblasts and lymphocytes enter some acini and destroy them. Especially in one piece do we find much vacuolation, deformation, and loss of nuclei; a great part of the transplant is entirely necrotic, other parts are dying. However, some relatively well preserved acini can still be found. At later periods mainly necrotic tissue is present. Some acini may be shrunken, other thyroid tissue is entirely dead. But as late as 18 days following transplantation there may be present a small amount of degenerating thyroid tissue which has not yet become entirely necrotic.

The first mitoses were seen 5 days after transplantation. After homoiotransplantation mitoses appear at earlier stages. There is, therefore, from the beginning a depression in mitotic proliferation after heterotransplantation. This inhibition is quite marked also in the following period; 6, 7, and 8 days after transplantation no definite mitoses were found. However, 9, 10, and 11 days following heterotransplantation a limited number of mitoses appeared. 11 days was the latest date at which they were found. The heterotoxins produce, therefore, a marked diminution in the number of mitoses in the transplanted thyroid.

The character of the vascularization of the heterotransplants is of great importance. 3 and 4 days after transplantation capillaries are found in the thyroid between the acinus cells. At 5 and 6 days fibroblastic tissue with capillaries can be seen around the grafts. From here a few vessels may penetrate between acini. Occasionally there is a specimen in which in certain places the vascularization between the acini is more plentiful. Such was the case in a specimen taken out 7 days after transplantation. From 8 to 14 days after transplantation there are usually a few capillaries between acini, but on the whole the lack of a satisfactory vascularization is quite apparent. The stroma is characterized by its lack of a good blood vessel supply.

The behavior of the connective tissue towards the heterotransplants is a determining factor in the result of the transplantation. At early stages, 5 and 6 days after transplantation, we find fibroblastic tissue with capillaries around the graft; some fibroblasts enter between the acini at various places and supply a stroma. Wherever fibroblasts are growing in larger number between the acini, they may enter the latter and destroy them. This is the condition found in the first 8 days. Mitoses are only rarely seen in the fibroblasts. In the following period, from the 9th to the 15th day, fibroblasts occasionally penetrating between and into acini are still found, but the fibrous character of the connective tissue around the acini is much more marked. This is, indeed, a noticeable effect of heterotransplantation. The stroma is, on the whole, densely fibrous or fibrillar and poor in vessels. This stroma compresses a considerable number of acini and exerts an injurious effect upon them. While fibroblasts thus

continue to break into the thyroid at various places, this infiltration of the interacinar spaces is in general not very extensive so long as there is living thyroid tissue present. Even under conditions of heterotransplantation the thyroid tissue exerts a certain inhibiting influence on the connective tissue of the host; the inhibition on the part of the parenchyma is greater than that presented by necrotic tissue. Thus the organization of the necrotic center is mainly effected by connective tissue growing into it at places where the thyroid ring is incomplete, while at places where the necrotic material adjoins the inner aspect of the thyroid the organization is much delayed.

Marked infiltration of the transplants by lymphocytes is not seen. Wherever there is much fibroblastic proliferation around the transplant and between the acini there may be small numbers of lymphocytes accompanying the fibroblasts. Towards the latter part of the 1st week and during the 2nd week lymphocytes may participate in the destruction of acini to a limited degree. In the 2nd week marked lymphocytic infiltration in the surrounding capsule at some distance from the thyroid proper may occasionally be found; lymphocytes may also sometimes penetrate into some adjoining furrows reaching into the transplant. But a marked collection of lymphocytes around and in the thyroid acini, such as is characteristic of homoiotransplantation, is not observed in heterotransplants. Even where the supply of blood vessels between the acini happens to be somewhat more extensive, a marked lymphocytic infiltration is not observed.

Lymphocytic infiltration may also be somewhat more marked in the dense fibrous tissue which is the result of the organization of necrotic material. Here again living thyroid tissue is absent. This organization of necrotic material takes place in two stages, the definite organization by a vascular connective tissue rich in fibroblasts and lymphocytes being preceded by the provisional production of hyaline material, representing a mixture of necrotic material and fibroplasm.

Ducts lined by squamous cell epithelium may temporarily survive after heterotransplantation.

In conclusion we find that the heterotoxins injure the transplanted cells directly and make them thereby susceptible to additional injurious influences, such as lack of good vascularization, fibrous character of the stroma causing compression of acini, and direct invasion and

destruction of a limited number of acini by fibroblasts and lymphocytes. These factors lead to a constant dying of transplanted parenchyma which is not compensated for by any appreciable new formation of tissue. The primary factor in the destruction of the heterotransplant is the injurious effect of the heterotoxins. They change the metabolism of the transplanted cells which now exert an effect upon the blood vessels, connective tissue cells, and lymphocytes which differs in certain respects from that exerted by normal as well as auto- and homoiotransplanted cells. These secondary reactions contribute to the destruction of the heterotransplants.

SUMMARY.

1. After transplantation of the thyroid of the guinea pig into rats there is a primary injury of the transplant, noticeable as early as 3, 4, and 5 days after transplantation. The tissue is less resistant, and it is preserved only under the best conditions. The number of mitoses is much diminished in the transplant, but they may appear even as late as 9 days after transplantation. This was also the latest time at which living tissue was found. Epithelium is best preserved in the neighborhood of growing fibroblastic tissue, and growing epithelium attracts fibroblasts. Few fibroblasts grow between acini, and they have a tendency to form fibrous tissue. Dense fibrous tissue compresses acini and contributes to their destruction. The vascularization of the graft is very poor, but some capillaries may penetrate between acini. The collection of lymphocytes around acini is only casual and may be found where fibroblasts are active and especially around the blood vessels in the capsule of the graft. On the whole, heterotransplanted tissue does not attract lymphocytes to any marked extent. Connective tissue and lymphocytes contribute only secondarily and to a minor extent to the destruction of the heterotransplant.

2. After transplantation of the thyroid of the rabbit into rats the last mitoses are found 9 days after transplantation; living acini are observed as late as 11 days after transplantation, and ducts of squamous epithelium even somewhat later. Different kinds of tissue seem to show a different degree of resistance to the action of hetero-

toxins. The difference in the resistance to heterotoxins corresponds to the difference in the resistance of various tissues to other kinds of injurious influences. The thyroid of rabbits is, on the whole, better preserved in the rat than the thyroid of the guinea pig; there is also more mitotic activity in the rabbit thyroid.

The reaction of the tissues of the host towards heterotransplanted rabbit thyroid in principle is similar to the reaction to heterotransplanted guinea pig thyroid.

3. After transplantation of rabbit thyroid into guinea pigs well preserved thyroid is found not later than 8 days, while the last mitoses appear 6 days after transplantation. The number of mitoses is very small. The host tissues again behave in a manner characteristic of heterotransplantation. It is probable that slight infections and the presence of polynuclear leucocytes are responsible for the somewhat inferior results in this kind of heterotransplantation.

4. After transplantation of cat thyroid into the rat, signs of degeneration in the transplanted acini appear at the end of the 1st week; they increase during the 2nd week. The last well preserved acini are found at 14 days; a few degenerating acini are still visible as late as 18 days after transplantation. Almost all pieces are entirely necrotic in the 3rd week. Mitoses are only found 5, 9, 10, and 11 days after transplantation, and they are present in a limited number. There is a decided lack of good vascularization in the transplants; it is especially noticeable in the 2nd week after transplantation. Fibroblasts penetrate at various places between the acini and occasionally together with a few lymphocytes may destroy some of them. From the 9th day on the presence of fibrous tissue around the acini is noticeable. It compresses the acini and thus contributes to their destruction. Even the heterotransplanted thyroid exhibits a restraining influence on the connective tissue which is greater than that presented by dead material. The behavior of the lymphocytes towards heterotransplanted thyroid of the cat is similar to that towards other heterotransplanted thyroid.

CONCLUSIONS.

After heterotransplantation of thyroid of various mammalian species into the rat and guinea pig, the latest period at which living tissue is found varies between 8 and 14 days. Exceptionally dying tissue may be found somewhat later. The last mitoses are observed between the 9th and 11th days in cases in which the tissue is found completely necrotic between the 9th and 14th days, and on the 6th day when the tissue is found dead 8 days after transplantation.

The heterotoxins injure the transplanted tissue directly and primarily and thus make it more susceptible to additional injurious influences, such as lack of good vascularization, fibrous character of the stroma, and direct invasion and destruction of a limited number of acini by fibroblasts and lymphocytes. Especially noticeable is the slight reaction of the lymphocytes towards the heterotransplants as compared with their strong reaction towards homoiotransplants.

A constant destruction of heterotransplanted parenchyma is not compensated for by any marked new formation of tissue. The action of the heterotoxins changes the metabolism of the transplanted tissue in such a way that the reaction of the various host tissues (vessels, fibroblasts, lymphocytes) towards the transplanted parenchyma is altered.

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